Stress and stationary phase characteristics in cell wall defective strains of Saccharomyces cerevisiae

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Submitted by Suzanne Bowen
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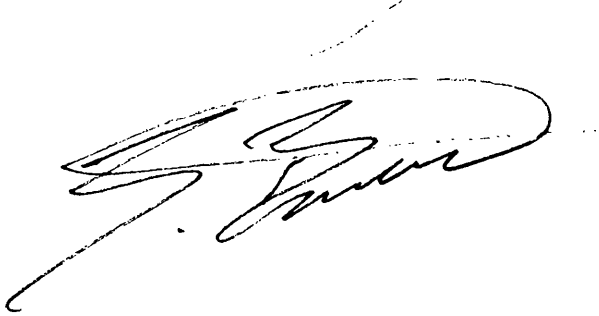

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Dedicated to Yvette and Justin.

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Abbreviations.

Ac	Acetate
CFW	Calcofluor White
CWH	Calcofluor White Hypersensitive
CWP	Cell wall protein
DDT	Dithiothreitol
dH_2O	Distilled water
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetate
ER	Endoplasmic reticulum
FGM	Fermentable growth medium
gDNA	Genomic DNA
GFP	Green fluorescent protein
Glc	Glucose
GN	Glucosamine
GPI	Glycosylphosphatidylinositol
GS	Glucan synthase
HA	Haemagglutinin (epitope tag)
HSE	Heat shock element
HSF	Heat shock transcription factor
hsp	Heat shock proteins
kre	K1 killer resistant
LB	Luria broth
Man	Mannose
MIPS	Martinsried Institute for Protein Sequences
NTPs	Nucleotide triphosphates
ORF	open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PIR	Protein containing internal repeats

PKC	Protein kinase C
PMSF	Phenylmethylsulphonyl fluoride
PVDF	Polyvinyl-difluoride
RNA	Ribonucleic acid
RT	Room temperature
SC	Synthetic complete
SDS-PAGE	Sodium dodecyl sulphate poly-acrylamide gel electrophoresis
SEM	Scanning Electron Microscopy
SGD	*Saccharomyces* genome database
STREs	Stress response elements
SZB	Sorbitol and zymolyase buffer
TE	Tris and EDTA
TFA	Trifluoroacetic acid
TSB	Transformation buffer containing DMSO
YNB	Yeast nitrogen base
YPD	Yeast peptone dextrose media

Gene Symbols used in this study.

ARD	Arrest defective
ATH	Acid trehalase
BCK	Bypass protein C kinase
BCY	Bypass of Cyclic AMP Requirement
CDC	Cell division cycle
CHS	Chitin synthase
CLB	Cyclin B
CLN	Cyclin
CRS	Copper-resistant suppressor
CUP	Copper
CTR	Copper transporter
CWP	Cell wall protein
CWH	Calcofluor-White hypersensitive
ERD	ER retention defective
FET	Iron transporter
FKS	FK506 Hypersensitive
FLO	Flocculation
FRE	Ferric reductase
GAS	GPI-anchored surface protein
GCS	Growth cold sensitive
GLC	Glycogen deficient
GLR	Glutathione oxidoreductase
GRR	Glucose repression
GSP	GTP binding protein
HOG	High osmolarity glycerol
KRE	K1 Killer resistant
MNN	Mannosylation deficient
MPK	Mitogen-activated protein kinase homolog
NTH	Neutral trehalase
PBS	Polymyxin B sensitive

PDE	Phosphodiesterase
PIR	Protein containing internal repeats
PMT	Protein-O-mannosyltransferase
PKC	Protein Kinase C
PLC	Phospholipase C
PMR	Plasma membrane ATPase related
PTC	Serine/threonine protein phosphotase
RAS	Ras homologue
ROD	Resistance to *o*-dinitrobenzene
SEC	Secretory mutation
SED	Suppressor of the erd2 deletion
SKN	Synthetic lethal kinase
SLG	Slow growth
SLT	Suppressor of lytic phenotype
SLY	Supressor of *YPT1* loss
SNF	Sucrose non fermenting
SNZ	Stationary phase protein
SOD	Super oxide dismutase
SPA	Spindle pole antigen
TIP	Temperature inducible protein
TPK	Protein kinase
TPS	Trehalose-6-phosphate synthase
VPS	Vacuolar sorting protein
WSC	Cell wall and integrity stress component
YPT	Yeast Protein Two

Abstract

Stationary phase has been generally defined as a physiological state co-ordinated with cell cycle arrest that allows cells to remain viable during prolonged periods of starvation. This involves biophysical modifications that include changes to the cell wall. One of the most notable changes to the cell wall is an increased level of glucanase soluble cell wall proteins (CWPs) accompanied by a higher incidence of *N*-glycosylation. The contribution of the cell wall to the viability of cells during stationary phase is assessed in this study.

Calcofluor White (CFW) is a cell surface, fluorescent dye that binds to nascent chains of chitin through hydrogen bonding and dipole interactions. Certain defects in cell wall assembly render the cell hypersensitive to CFW. The strains screened in this study were from a library of cell wall mutants obtained by selecting for CFW sensitivity. Sixty-three monogenic Calcofluor White hypersensitive (*cwh*) mutants were isolated from 5,000 chemically mutated cells and classified into 53 complementation groups. These strains were then categorised further into subgroups according to cell wall mannose/glucose ratio and by other phenotypic tests involving drugs that are known to affect cell surface synthesis and regulation.

Fifty strains from the *cwh* library were screened for loss of viability in long term stationary phase. Twenty of these had reduced viability under experimental conditions. Several have other phenotypes such as Zymolyase sensitivity or *K1* killer toxin resistance (*kre*). *Kre* cells of *S. cerevisiae* usually have a reduced level of β1,6-glucan in the cell wall polysaccharide matrix caused by a mutation

that affects cell wall construction or the secretory pathway. β1,6-glucan is involved in anchoring CWPs to the cell wall. As the *kre* strains isolated had previously quantified levels of β-glucan, chitin and mannoproteins they were selected to monitor the influence of cell wall composition in stationary phase.

The viability of five *kre* yeast strains, *cwh13/erd1, cwh30/kic1, cwh41/gls1, cwh47/ptc1* and *cwh48/kre6*, was assessed for entry and exit from stationary phase. Stress characteristics such as intracellular trehalose content and resistance to Zymolyase and Cu^{2+} were also determined. The strain with the lowest viability in stationary phase, *cwh48/kre6*, had the greatest resistance to Cu^{2+}. The strain with the greatest Zymolyase resistance, *cwh30/kic1*, was found to have the highest intracellular trehalose and CWP content. In contrast, strains that were sensitive to Zymolyase, *cwh13/erd1, cwh47/ptc1* and *cwh48/kre6*, had reduced levels of intracellular trehalose and CWPs. The *cwh30/kic1* strain induced stress characteristics while growing on a fermentable carbon source under optimal conditions. These findings reveal that a decrease in levels of Kic1p produce a defective cell wall and induce stress response characteristics. In comparison, *cwh47/ptc1* and *cwh48/kre6* cells do not adopt the characteristics associated with stationary phase and therefore have a reduced viability when compared to the *cwh30/kic1* and wild type AR27 strains. A model to illustrate the involvement of *cwh30/kic1* and *cwh47/ptc1* in stress response pathways is presented in this work.

As yeast cells enter stationary phase, transcription of specific GPI anchored CWPs is increased. CWPs have been implicated in playing a vital role towards cell viability and re-proliferation during stationary phase. They are anchored to the β1,3-glucan cell wall matrix by a complex series of linkages involving β1,6-

glucan and chitin. To investigate the influence of reduced β1,6-glucan on the level of CWPs retained by the cell during stationary phase, a cell wall protein associated with stationary phase, Sed1p, was quantified in *kre* strains. The expression of *SED1* mRNA increases 2.7 fold in stationary phase cells when compared to exponentially dividing cells. The *kre* strains, *cwh13/erd1*, *cwh30/kic1*, *cwh41/gls1*, *cwh47/ptc1* and *cwh48/kre6*, were transformed with an HA-epitope tagged *SED1* gene. Amongst these strains *cwh48/kre6* was found to be defective in incorporating Sed1p into the cell wall. The molecular weight of the protein found in cytoplasmic extracts of *cwh48/kre6* was a similar size to that found in other *kre* cells and the control. The gene product of *cwh48/kre6* is thought to function as a mannosyl transferase that is involved in the modification of *N*-chains and GPI-anchors. As *cwh48/kre6* also has the lowest viability in stationary phase, this implies that a cell wall capable of supporting increased levels of CWPs assists survival in long term stationary phase. Overall this work demonstrates that a cell wall defective strain without the ability to produce or sustain stress-associated characteristics loses viability in long-term stationary phase.

Chapter 1

Introduction

Saccharomyces cerevisiae is an ascomycetous, unicellular fungus, that is exploited in several industrial processes for biomass production and for the by-products it produces as a consequence of fermentative growth. *S. cerevisiae* is also extensively studied in developmental research as a model cell system because it shares several characteristics with higher eukaryotes. However, although it is similar in many ways to plant cells, it differs from animal cells by the presence of a cell wall and by a unique form of vegetative reproduction through asymmetrical budding. In comparison with other fungi, *S. cerevisiae* also has many attributes that make it an ideal organism to study molecular genetics; it grows rapidly, is easy to culture, is non-pathogenic and has a fully sequenced genome. In addition, it has both haploid and diploid life-cycles which allow mutations to be created and analysed by classical genetic approaches (Hall and Linder, 1993).

1.1 Growth conditions and cell integrity.

As in several other microorgansims, yeast species are evolutionary successful because they have a number of adaptive mechanisms to avoid stress. In response to nutrient limitation, *S. cerevisiae* has the ability to alter morphology and patterns of growth to increase chances of survival (Fuge and Werner-Washburne, 1997). For instance, in response to nitrogen limitation, diploid cells can form unipolar pseudohyphae or undergo meiosis to produce four haploid cells

(ascospores) that differ chemically and morphologically from vegetative cells (Cid *et al.*, 1995). Under nutrient limitation vegetative diploid and haploid cells can also enter a quiescent state known as stationary phase. Once stationary phase is entered, cells have a greater resistance to environmental extremes and can remain dormant for several months while retaining the ability to resume growth when nutrient levels increase or conditions improve (Fuge and Werner-Washburne, 1997).

Mutations that effect cell viability during starvation can occur in several biophysical pathways that regulate cellular processes (Table 1.1). The rest of this chapter is concerned with describing some of these processes, in particular, growth, metabolism, resistance to stress, cell integrity and cell wall construction.

1.1.1 *Yeast growth.*

Yeast growth, in complete media with a fermentable carbohydrate (e.g., glucose, fructose or mannose) as a source of carbon, follows a series of well defined phases (Fig.1.1). Following a brief lag period, cells divide exponentially in

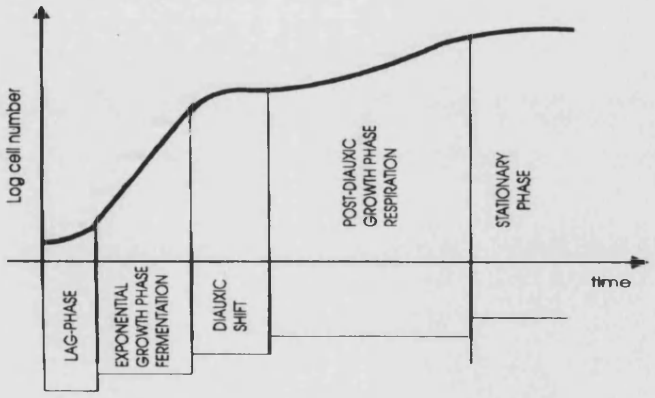


Fig 1.1. Growth phases in *S. cerevisiae*. Following a brief lag phase, cells grow exponentially on a fermentable carbon source (e.g., glucose). When glucose is exhausted, cells enter the diauxic shift and change metabolically to allow them to grow on a non-fermentable carbon source (e.g., ethanol). When all nutrients are depleted cells become quiescent as they enter stationary phase.

Table 1.1. Genes and gene products that influence sensitivity to starvation in *S. cerevisiae* (information obtained from Martinsried Institute for Protein Sequencing (MIPS) database, April 2000).

ORF	Gene	Gene product
YAL062w	*GDH3*	NADP-glutamate dehydrogenase
YBR217w	*APG12*	component of the autophagic system
YCR009c	*RVS161*	similarity to human amphiphysin and Rvs167p
YDL212w	*SHR3*	ER membrane protein
YDR388w	*RVS167*	reduced viability upon starvation protein
YFL038c	*YPT1*	GTP-binding protein of the *rab* family
YGL173c	*KEM1*	multifunctional nuclease
YGL180w	*APG1*	essential for autophagocytosis
YGL227w	*TIN1*	TOR inhibitor
YHR030c	*SLT2*	ser/thr protein kinase of MAP kinase family
YHR171w	*APG7*	component of the autophagic system
YIL033c	*SRA1*	cAMP dependent protein kinase, regulatory subunit
YJL095w	*BCK1*	ser/thr protein kinase of the MEKK family
YJR090c	*GRR1*	required for glucose repression
YLL039c	*UBI4*	ubiquitin
YLR337c	*VRP1*	verprolin
YLR399c	*BDF1*	sporulation protein
YNL066w	*SUN4*	cell wall protein with homology to β-glucosidase
YNL138w	*SRV2*	adenylate cyclase-associated protein, 70kDa
YOL081w	*IRA2*	GTPase-activating protein for RAS proteins
YOR231w	*MKK1*	ser/thr protein kinase
YOR360c	*PDE2*	high affinity phosphodiesterase
YPL140c	*MKK2*	protein kinase of the MEK family
YPL149w	*APG5*	involved in autophagy and nutrient starvation
YPL154c	*PEP4*	aspartyl protease
YPR185w	*APG13*	protein required for the autophagic process

fermentative growth. During fermentation yeast uses organic substrates anaerobicaly as electron donors, electron acceptors and a carbon source. The carbon source enters the glycolytic pathway and produces ATP by substrate phosphorylation (Fig.1.2). Glycolysis is a sequence of enzyme-catalysed reactions that converts glucose to pyruvic acid (Equation 1.1). During this process, carbohydrates are gradually converted into ethanol and CO_2.

Equation 1.1 Glucose $\rightarrow$ glycolysis $\rightarrow$ Pyruvate + 2ATP + 2NADH + H^+

As carbohydrate becomes limiting, cells enter a second lag phase, the *diauxic shift*, where they switch metabolically from fermentation to respiration. Cells then resume growth using a non-fermentable carbon source such as ethanol, acetate or other by-product of the fermentative growth phase. Respiring cells metabolize their energy source through the citric acid (TCA) cycle where ATP is produced by oxidative phosphorylation (Fig. 1.3). The overall affect of the citric acid cycle is to produce $2CO_2$, 3NADH, 1 $FADH_2$, $4H^+$ and 1 GTP from a single molecule of pyruvate. When all carbon sources are exhausted, cells enter the non-dividing state of stationary phase. In growing cultures of *S. cerevisiae* respiration accounts for 3-20% of sugar catabolised, in resting cells this increases to 25-100% (Lagunas *et al.*, 1982). *S. cerevisiae* can also grow in media where a non-fermentable source such as glycerol replaces the fermentable carbohydrate.

1.1.2 *Population Growth.*

When cells are in lag phase the growth rate is equivalent to zero. Cells not only enter lag phase during normal growth but also when growth conditions demand an alteration in metabolism, for instance, due to high

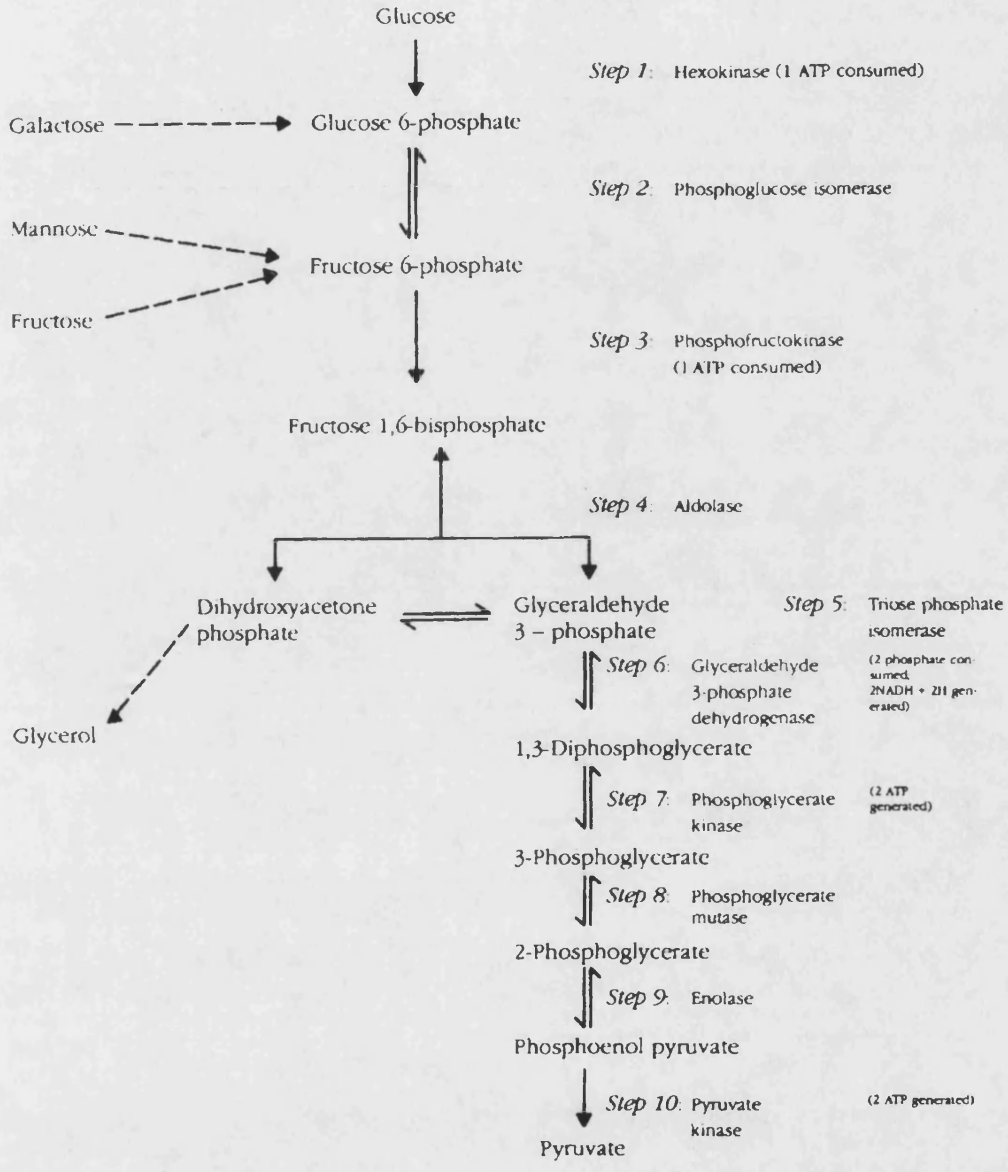


Figure 1.2. Glycolysis in *S. cerevisiae*. Glucose is phosphorylated using two ATPs to produce fructose 1,6 biphosphate. Further degradation eventually generates four ATPs and the formation of two molecules of pyruvic acid (from Walker, 1998).

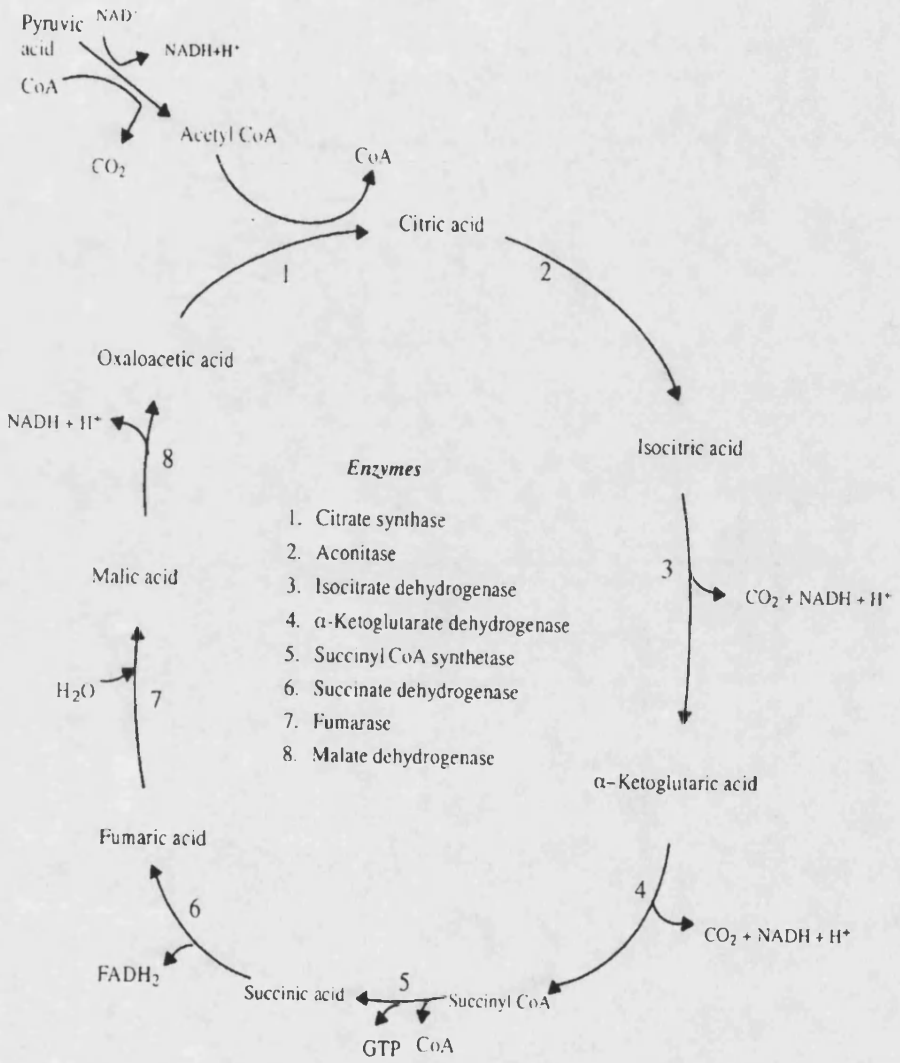


Figure 1.3. Citric acid cycle in yeast. Produces $2CO_2$, 3NADH, $1FADH_2$, $4H^+$ and 1GTP from one pyruvate molecule (from Walker, 1998).

osmolarity or temperature. During lag phases, cells synthesise enzymes and ribosomes required for growth at an accelerated rate (Walker,1998). In accelerated growth, yeast biomass can be expressed differentially by the following equation (Equation 1.2).

Equation 1.2 $dx/dt = \mu x$

Where the change in yeast biomass, *dx/dt*, in time, *t*, can be determined by the value of the specific growth rate, μ, in relation to biomass, *x*. The logarithmic growth of cell doubling in exponential phase can be expressed in terms of maximum specific growth rate (μ_{max}) (Equation 1.3) which can be integrated in to the fundamental equation for exponential growth (Equation 1.4).

Equation 1.3 $dx/dt = \mu_{max}x$

Equation 1.4 $x_t = x_0e^{(\mu_{max}t)}$

Following exponential phase yeast growth decelerates and begins to correlate with substrate limitation. Limitation of growth can then be represented by the Monod equation (Equation 1.5). During stationary phase yeast growth ceases and the population size remains constant ($\mu = 0$) until cells begin to lyse. Loss of viability in cells can be represented differentially by replacing growth rate (μ) by death rate, *k* (Equation 1.6).

Equation 1.5 $$\mu = \mu_{max} \frac{[S]}{[S] + K_s}$$

Equation 1.6 $-dx/xt = kx$

1.1.3 *Cell division cycle.*

At the cellular level, reproduction in *S. cerevisiae* is mainly achieved by budding. When cells reach a critical size, the initiation of budding coincides with the synthesis of DNA. Cell wall constructing enzymes such as chitinases and glucanases are then thought to be directed by localised actin bound to plasma receptors to a weakened area in the cell wall (Drubin *et al.*, 1993). Chitin and septins encircle the junction between mature cell and the newly created bud to allow cytoplasm to be extruded into the newly formed cell. Following cell separation the chitin ring forms the perimeter of the bud scar. This can be visualised by electron microscopy (Fig.1.4) or by staining the cells with the fluorescent dye Calcofluor White (Fig.1.5). Septins form the cleavage plane which bisect the spindle axis at cytokinesis.

The usual way to assess the physiological state of a population is to determine the budding index (BI). Rapidly dividing cells will have a high BI of above 50% while a population which is residing in stationary phase may have a budding index of less than 20%. The cell cycle in actively dividing cells of *S. cerevisiae* has four defined phases between the division of the mother cell and subsequent division of the daughter cell. These phases are; pre-synthetic gap (G_1 phase), DNA synthesis (S phase), post-synthetic gap (G_2 phase) and mitosis (M phase). Stressed cells temporarily exit the cell cycle at G_1 and enter a quiescent non-dividing state in which they are physiologically distinct from actively dividing cells. This state is also known as G_0 (Fig 1.6). The majority of a cell life-cycle can be spent residing in G_0.

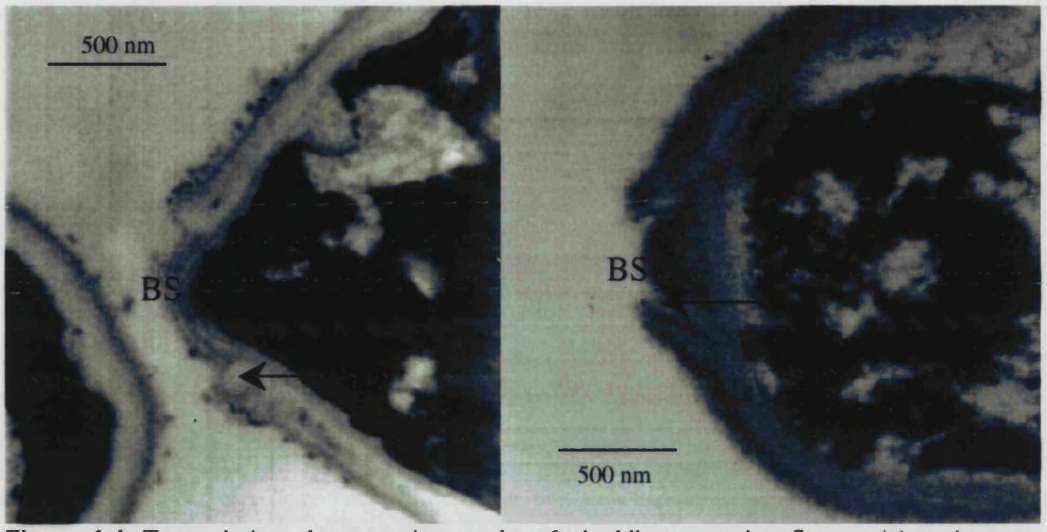
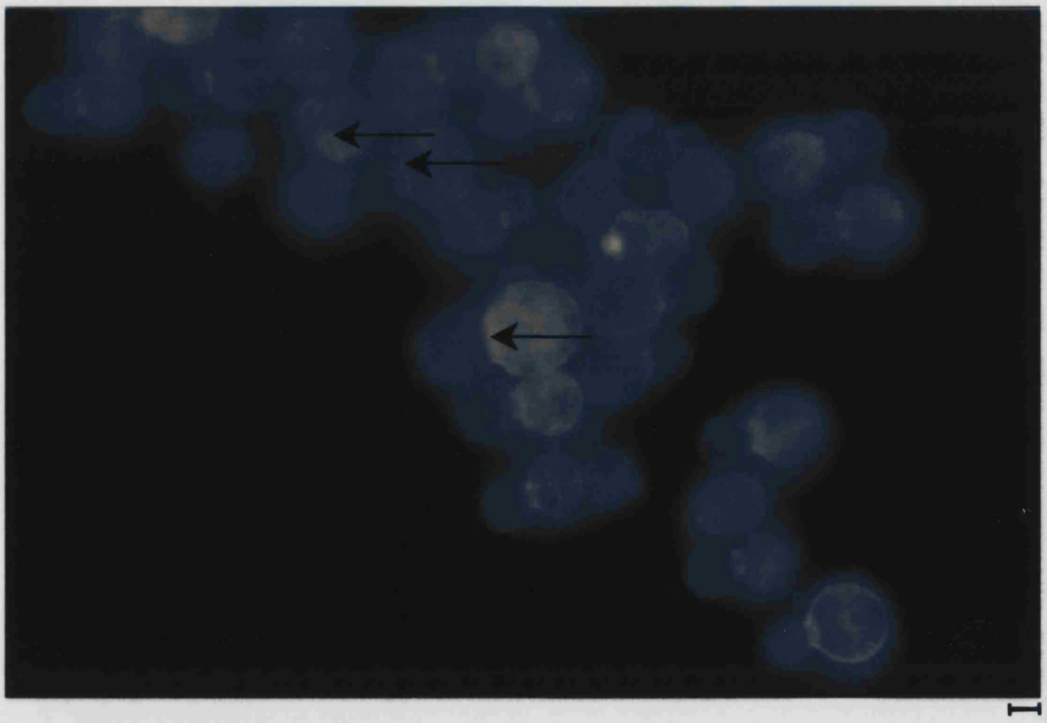


Figure 1.4. Transmission electron micrographs of budding scars in *S. cerevisiae*. Arrows indicate chitin deposition. BS, Bud scar.

Figure 1.5. Budding *S. cerevisiae* cells treated with calcofluor white fluorescent (CFW) dye. When bound to chitin CFW fluoresces in UV light. Arrows indicate chitin deposition in septum and budding scars. Scale bar represents 1 μm.



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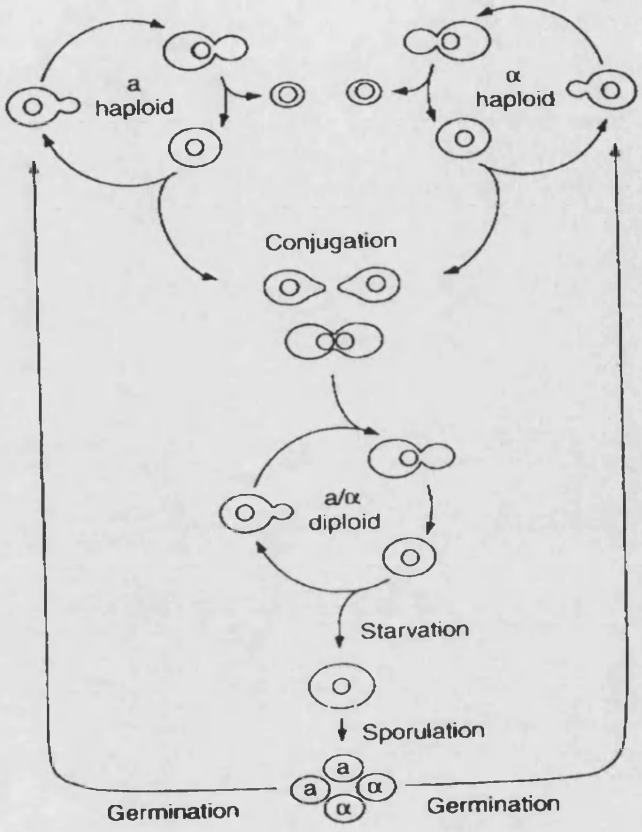

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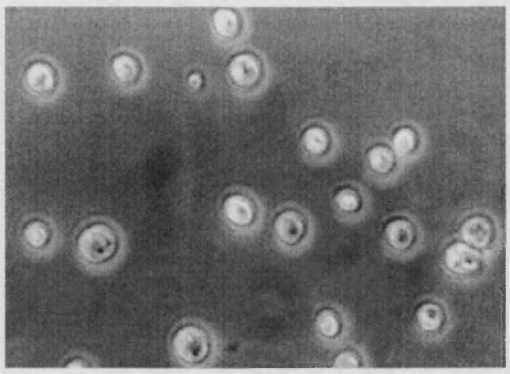
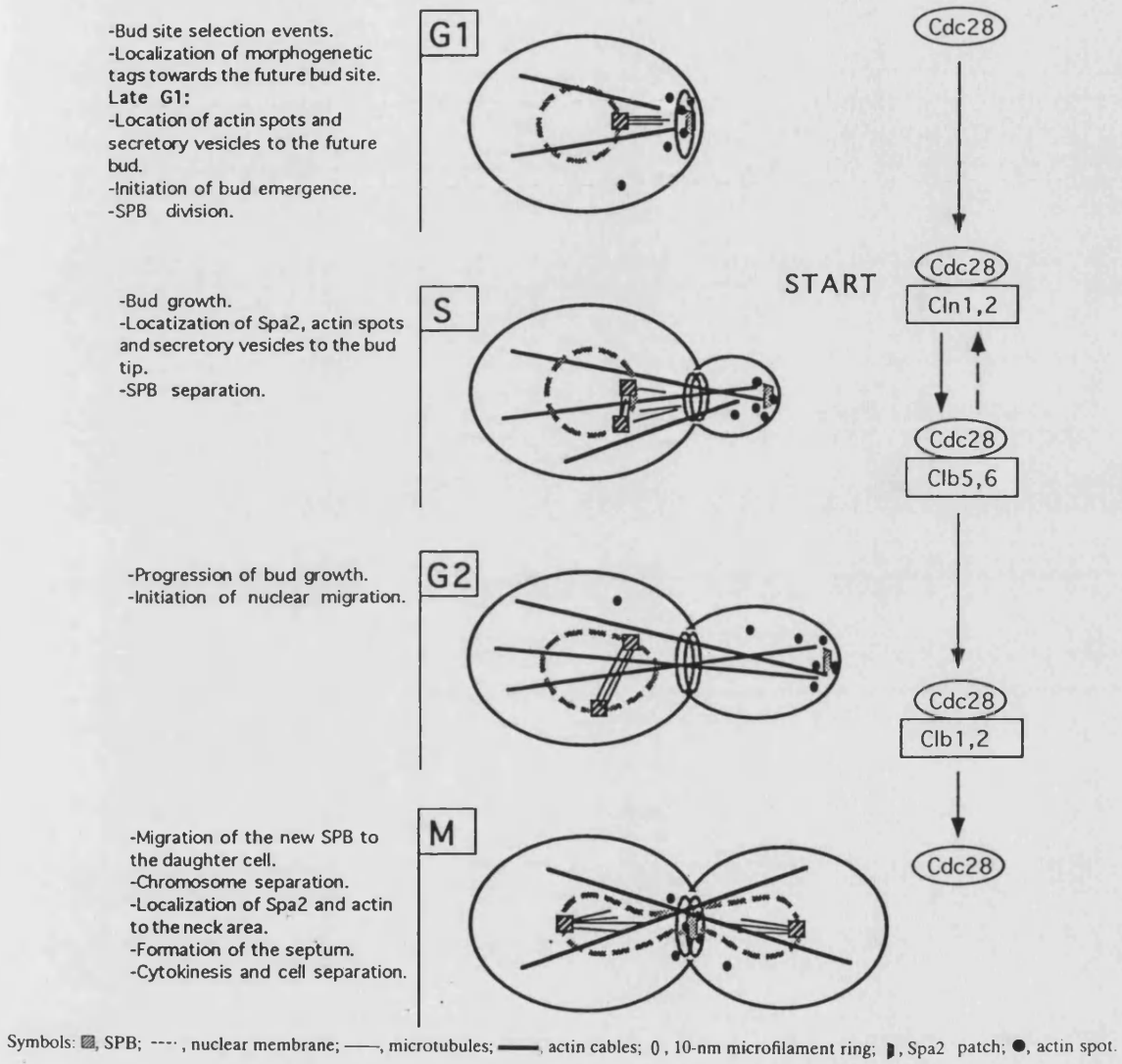


Figure 1.6. Life-cycle in *S. cerevisiae* (a) (from Murray and Hunt, 1993). Haploid cells either reproduce by budding or two opposing mating types conjugate to form diploid cells. Under starvation diploid cells either sporulate or enter stationary phase (b). In stationary phase cells become unbudded and adopt stress-resistant characteristics.

The various stages of cell division have been analysed by using cell cycle division (*cdc*) mutants (Reed, 1980; Pringle and Hartwell, 1981). Morphogenesis is intrinsically linked to the cell cycle (Fig 1.7). Bud site selection, bud emergence and localisation of the actin cytoskeletal apparatus occur in G_1. Bud growth occurs from late G_1 through to S and is completed in G_2; this stage involves changes in actin localisation from an apical position to a more diffuse distribution. Cytokinesis and subsequent cell separation involve the redistribution of actin to the septum area and occurs after mitosis. Successful bud formation demands synchrony between nuclear and cortical events as many of the bud formation stages are performed independently of nuclear division. This has been demonstrated by mutations that block the early stages of budding (*CDC24* and *CDC42*) and still allow nuclear division to take place (Hartwell *et al.*, 1974). Spindle pole body (SPB) duplication and cell division are thought to be induced by the same signal as they both start simultaneously in late G_1 (Pringle and Hartwell, 1981). These events are preceded by the binding of a protein kinase, encoded by *CDC28*, to regulatory proteins called cyclins, encoded by *CLN* genes (Reed, 1980). This coincides with cell division leading to bud emergence and SPB duplication (Johnston and Lowndes, 1992). The kinase encoded by *CDC28* is then thought to bind with other cyclins encoded by *CLB3, CLB4, CLB5* and *CLB6* to perform several other functions during the progress of the cell cycle through to S phase. Cdc28p is not thought to be involved with the cytoskeletal rearrangements leading to polarised secretion and bud initiation (Madden and Snyder, 1992). During G_1, microtubules extend from the SPB towards the future bud site. When the bud emerges, actin, Spa2p, Rho proteins, calmodulin, and secretory vesicles localise at the bud tip (Adams and Pringle,



Symbols: ▨, SPB; ----, nuclear membrane; ——, microtubules; ▬▬, actin cables; 0, 10-nm microfilament ring; ◗, Spa2 patch; ●, actin spot.

Figure 1. 7 Cell division cycle in relation to cell surface growth events and the binding of protein kinase Cdc28p to cyclins (Cid *et al.*, 1995).

1984). These become diffused throughout the cell wall until after mitosis when they migrate to the septum to become involved in the synthesis of new cell wall inside the chitin ring (Lew and Reed, 1993). The depolarisation of actin at the bud tip is a cell cycle function that is associated with the activation of the Cdc28 kinase by other cyclins encoded by *CLB1*and *CLB2*. Inactivation of Cdc28p after mitosis preludes the relocalisation of the actin cytoskeletal to the septum.

1.2 Cell metabolism and accumulation of storage carbohydrates.

1.2.1 *Glucose repression.*

Yeast grows preferentially by fermentation on a fermentable carbon source such as glucose. When glucose becomes exhausted a signalling cascade is initiated in the cell. Glucose limitation activates the protein kinase Snf1p (Hubbard *et al.*, 1992). This results in the transcription of a number of genes encoding enzymes that are involved in the utililsation of a non-fermentable carbon source such as ethanol or acetate and causes the cell to switch to a different metabolic pathway. This transition phase is known as the main glucose-repression or catabolite repression pathway (Gancedo, 1992; Trumbly, 1992). Repression and inactivation may not be mutually exclusive mechanisms. Both may be functioning when growth occurs on certain substrates, such as maltose (Entian and Zimmermann, 1982).

1.2.2 *Glycogen metabolism.*

Glycogen is a polysaccharide consisting of a highly branched polymer of glucose. It is synthesised in glucose-grown cells when the fermentable carbon source begins to diminish. During starvation, stored glycogen is fermented endogenously

to contribute to cell maintenance by supplying ATP needed for continued cell viability. Elongation of β-1,4 glucosyl linkages involves the activity of a glycogen synthase, encoded by *GSY1* and *GSY2* (Farkas *et al.*, 1991). Addition of the branching β-1,6 linkages are catalysed by an enzyme encoded by *GLC3* (Rowen *et al.*, 1992). Glucan synthase is deactivated by phosphorylation involving an unidentified protein kinase. It is believed that the activity of this protein kinase could be cAMP dependent because levels of cAMP rise consecutively with glucan synthase deactivation (Dickinson, 1998). Glycogen is degraded into glucose and glucose-1-phosphate by glycogen phosphorylase and a debranching enzyme.

1.2.3 *Trehalose mobilisation.*

Trehalose is thought to be a storage carbohydrate because it accumulates in cells subjected to nutrient limitation (Werner-Washburne *et al.*, 1993; Fuge *et al.*, 1997; De Winde *et al.*, 1997). It is also thought to offer cells protection during certain adverse stress conditions such as in heat tolerance and against toxic chemicals (Nwaka and Holzer, 1998). The exact mechanism by which it offers protection has not yet been elucidated. Trehalose accumulation does not always correlate with resistance to stress (Nwaka and Holzer, 1998). Recently it has been discovered that trehalose may contribute to stress protection by preventing proteins from aggregating after they have been denatured by environmental influences (Singer and Lindquist, 1998).

Trehalose is a non-reducing disaccharide of glucose that occurs naturally in several organisms throughout nature (Elbien, 1974). The molecular biology of

trehalose and the trehalases in yeast has been extensively reported in a recent review by Nwaka and Holzer (1998). Trehalose is synthesised by the catalytic actions of a complex, trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, on the substrates UDP-glucose and glucose-6-phosphate. The majority of trehalose hydrolysis in yeast involves the actions of two enzymes, neutral trehalase, which has a maximal activity at pH 7, and acid trehalase which has a maximal activity at pH 4.5. Neutral trehalases, encoded by *NTH1* and *NTH2*, are localised in the cytosol where their activation involves the participation of cAMP, ATP and Mg^{2+}. Acid trehalase is encoded by *ATH1*, a permanently active enzyme located in vacuoles. The biological function of trehalase is to mobilise and control the concentration of trehalose by degrading it into two glucose molecules (Nwaka and Holtzer, 1998).

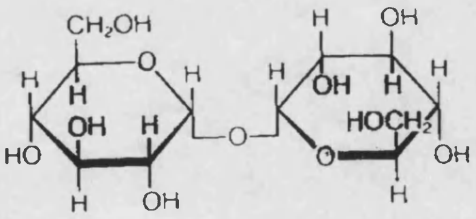


Figure 1.8. Trehalose structure. Trehalose is a disaccharide of glucose molecules linked by the α anomer of carbon 1.

Several studies have correlated a rise in intracellular trehalose with a specific stage in physiological development (Lillie and Pringle, 1980). These steps also coincide with a temporary cessation of cell division. For instance, when cells are undergoing respiratory adaptation (Panek and Matton, 1977), on entering stationary phase (Lillie and Pringle, 1980) and under various physical stresses such as changes in temperature (Kienle *et al.*, 1993) or osmolarity (Singh and Norton, 1991). These factors have led to the assumption that elevated levels of

trehalose act as a stress protectant. However, a number of studies have revealed that a high level of trehalose does not necessarily confer tolerance to some stress conditions. A mutant, deficient in the synthesis of the heat shock protein Hsp104, was found to have less resistance to temperature stress than the wild type strain even though there was no significant difference in trehalose levels between the two strains (Winkler *et al.*, 1991). Other studies have revealed that trehalose levels only appear to offer thermo-protection in yeast cells grown in a non-fermentable carbon sources but not those grown in fermentable carbon sources (Van Dijck *et al.*, 1995).

Expression of the neutral trehalase genes, *NTH1* and *NTH2*, increases under various conditions such as in response to temperature change and toxic chemicals (Nwaka and Holzer, 1998). These genes are also necessary for recovery of yeast cells following heat shock (Nwaka *et al.*, 1995). Deletion of trehalase genes affects growth on non-fermentable carbon sources. Trehalase mutants, *nth1* and *ath1*, have been found to grow poorly on glycerol when compared to a wild type strain (Nwaka *et al.*, 1995). Neutral trehalase is induced mainly in cells that are actively growing on glucose (Wiemken, 1990). The activity of neutral trehalase is reduced in yeast cells grown on a non-fermentable source such as glycerol. In conditions of high osmolarity, neutral trehalase activity is not increased (Zahringer *et al.*, 1997).

1.2.4 *Involvement of cAMP in stationary phase.*

When a fermentable sugar such as glucose is added to yeast cells starved of carbon, a rapid increase of transient cellular cAMP is induced. Levels of

intracellular cAMP reflect the growth conditions of the cells. The regulation of cAMP in *S. cerevisiae* is controlled by a complex pathway (Broach and Deschenes, 1990). The gene products of *RAS1* and *RAS2* act as stimulatory G proteins on adenylate cyclase, encoded by *CYR1/CDC35*. They are active in the GTP-bound form and inactive in the GDP-bound form. Exchange of GDP for GTP involves the gene product of *CDC25*. The Ras proteins are inactivated by intrinsic GTPase regulation which is stimulated by the products of *IRA1* and *IRA2*. Hydrolysation of cAMP involves a low-affinity phosphodiesterase and a high-affinity phosphodiesterase, encoded by *PDE1* and *PDE2*. cAMP then activates protein kinase A by binding to a regulatory subunit encoded by *BCY1*.

The cAMP pathway is known to be a regulator of stress resistance. Mutations in $RAS2^{VAL19}$ or *BCY1* lead to stress sensitivity and a lack of stored carbohydrate in stationary phase. Mutations that inhibit cAMP and temperature sensitive alleles of *CDC25* or *CYR1* (adenylate cyclase), arrest as metabolically inactive G_1 cells that are stress resistant even in rich medium. When genes that encode the regulatory subunit of the cAMP-dependent protein kinase (cAPK) are deleted, a mutant that is unable to survive nutrient limitations and extreme temperatures is created. Sporulation, glycogen metabolism and heat tolerance, have been found to be controlled independently of cAMP (Cameron *et al.*, 1988). *BCY1* encodes a regulatory subunit while *TPK1*, *TPK2* and *TPK3* encode catalytic subunits of cAPK. In contrast to wild type yeast cells, *bcy1*Δ strains do not accumulate glycogen, form spores or become resistant to heat shock under nutrient limitations (Peck *et al.*, 1997). Mutations of the gene encoding components of the cAMP pathway create similar phenotypes, such as, activation of *RAS* proteins

that stimulate adenyl cyclase or deletion of the genes that encode the cAMP phosphodiesterases. Mutant alleles of the *TPK* genes (*tpkw*) can suppress phenotypes created by mutations in the cAMP pathway.

1.3 Protein modification in the Secretory Pathway.

The molecular mechanisms involved in protein transport and secretion has been analysed by various thermosensitive secretory mutants (Novick and Scheckman, 1979). *SEC* genes have been identified which have specific functions in the translocation of proteins through the secretory pathway by isolating mutants that inhibit various stages of secretion (Novick *et al.*, 1980). These include translocation over the ER membrane (*sec61, sec62*), transport from ER to the Golgi apparatus (*sec12, sec13, sec23*), transport through the Golgi-tract (*sec7, sec14*) and exocytosis (*sec1, sec10, sec15*) (Novick *et al.*, 1980; Novick and Scheckman, 1979). Several of these mutations cause the accumulation of post-Golgi secretion vesicles. When the secretion vesicles were purified by gel filtration or density gradients they were found to contain glycoproteins destined for the periplasmic space, plasma membrane and cell wall (Harsay and Bretscher, 1995).

In the secretory pathway, membrane and secretory proteins are co-translationally inserted into the endoplasmic reticulum (ER) by an amino-terminal signal sequence (Montijn, 1996). Within the lumen of the ER the signal peptide is removed and proteins are further modified by proteolytic processing, folding and glycosylation (Helenius *et al.*, 1992). The newly modified proteins are packaged

into transport vesicles to be transported to the Golgi complex. The accurate mediation of proteins through the secretory pathway involves a number of target signals on luminal and membrane bound components (Helenius *et al.*, 1992). Resident luminal proteins are responsible for early protein modification. When luminal ER proteins reach the Golgi complex they receive characteristic carbohydrate modifications and are then recycled to the ER. ER retention involves a C terminal tetrapeptide sorting signal, HDEL, and a receptor, which is the *ERD2* gene product (Lewis *et al.*, 1990).

1.3.1 *Secretory mutants with reduced viability*

Several yeast strains, which lose viability in stationary phase, have mutations in genes required for protein secretion (Werner-Washburne *et al.*, 1993). Two examples are found in strains containing a mutation in *YPT1* and *PMR1*. *YPT1* encodes a small GTP binding protein that is associated with the Golgi apparatus. When cells are starved for nitrogen, Ypt1p is necessary for cell maintenance in un-budded cells. Cells are rescued by the over-expression of genes which encode synaptobrevin-like membrane proteins, *SLY2* and *SLY12*. *PMR1* also encodes a protein that is involved in transportion though the Golgi apparatus. The gene product of *PMR1* resembles a Ca^{2+} ion pump. The slow growth of this phenotype can be suppressed by the addition of calcium to the external media but this has been found to exacerbate loss of viability (Schmitt *et al.*, 1988).

1.3.2 *Glycosylation*

Glycosylation occurs when a carbohydrate moiety is covalently linked by the catalytic involvement of glycotransferases to a specific amino acid in a

polypeptide chain. Carbohydrates are either linked to the hydroxyl groups of serine, threonine or hydroxylysine (*O*-glycosidic linkage) or they are linked to the amide group of asparagine (*N*-glycosidic linkages). The initial stages of *N*-glycosylation follows a similar pathway in all eukaryotes whereas *O*-glycosylation differs between organisms.

The *N*-glycosylation of glycoproteins begins within the ER. The core oligosaccharide ($Glc_3Man_9GlcNAc_2$) is transferred, by a *N*-oligosaccharyl transfer complex, from a dolichol-pyrophosphate to the amide of asparagine in an Asn-Xxx-Thr(Ser) sequence. Once this transfer is completed the protein is further modified by the removal of glucose and mannose residues. Glucose residues are removed by glucosidases I and II. Glucosidase I cleaves a terminal α-1,2-linked glucose and glucosidase II cleaves two α-1,3-linked glucoses. *N*-chains are elongated with mannose residues in the Golgi complex. Elongation takes place either as core maturation, Man_9-$14GlcNAc_2$, and as outer chain glycosylation, $Man>50GlcNAc_2$.

In *S. cerevisiae*, *O*-glycosylation begins in the ER with the transfer of a mannose residue from dolichyl-phosphatase-mannose to threonine or serine residues. This transfer is catalysed by a *O*-mannosyltransferase encoded by the *PMT* genes (Gentzch and Tanner, 1996). Further elongation takes place in the Golgi complex by the addition of two mannose residues transferred from GDP-Man (Montijn, 1996). A third mannose residue is transferred by an α1,2-mannosyltransferase (encoded by *MNT1/KRE2*) which is also involved in *N*-glycosylation. In some

instances, a further two α1,3-linked mannose residues can be added by a catalytic reaction involving the *MNN1* gene product.

Following modification, secretion vesicles transport the proteins to the plasma membrane. Vesicles either contain proteins destined to the cell wall and plasma membrane or proteins destined for the periplasmic space (Walworth and Novick, 1987; Harsay and Bretscher, 1995). When vesicles reach the plasma membrane they fuse releasing proteins that then migrate to their final destination.

1.4 Nutrient starvation and Stationary phase.

During fermentative growth in glucose when the medium becomes exhausted of another elemental nutrient, such as nitrogen, phosphate or sulphur, cells adopt similar characteristics to cells in stationary phase. These characteristics are also observed in cells that are grown in non-fermentable carbon sources. When the medium is supplemented with the required nutrients it initiates a recovery response in both cells starved for nutrients and cells growing on non-fermentable carbon. Trehalose is rapidly mobilised by the post-translational activity of trehalase; this process precedes reproliferation and is independent of protein synthesis (Hirimburegama *et al.*, 1992).

1.4.1 *Physiological stationary phase characteristics.*

Stationary phase cells acquire physiological and morphological characteristics which distinguish them from exponentially dividing cells (reviewed in: Fuge and Werner-Washburne,1997; Werner-Washburne *et al.*, 1993; Werner-Washburne *et*

al., 1996). Observed under the microscope, stationary phase cells are rounded and unbudded and contain unreplicated DNA folded into condensed chromosomes. Transcription is reduced to below 0.3% and translation reduced to less than 10% of the levels found in exponentially growing cells. Cells become refractile, have thick, less porous cell walls and are more resistant to environmental stress, such as heat-shock. The cytoplasm becomes granular and accumulates carbohydrates such as glycogen and trehalose. Many of the characteristics associated with stationary phase are also observed in cells under stress, for example, exponentially growing cells that are heat shocked will also accumulate trehalose. To distinguish stationary phase cells from stressed cells, stationary phase has been generally defined as a physiological state co-ordinated with cell cycle arrest that allows cells to remain viable during prolonged periods of starvation (Werner-Washburne *et al.*, 1993).

1.4.2 Genetic pathways involved in stationary phase entry, residence and exit.

Entry into stationary phase involves the participation of several signal pathways. RAS cyclic AMP pathway is involved in cell metabolism. PI pathway has a role in nutrient sensing and the PKC pathway is involved in cell wall construction.

On entering stationary phase cells become transcriptionally repressed. This repression is carried out by topoisomerase I (Choder, 1991). DNA topoisomerase I catalyzes the relaxation of supercoiled DNA through a series of strand breakage and religation (Megonigal *et al.*, 1997). Cells that are topoisomerase I-deficient continue to transcribe genes in stationary phase (Choder, 1991). Certain mutations in the *TOP1* gene can lead to cell cycle arrest and cell death

(Megonigal *et al.*, 1997). Although in stationary phase changes occur in the physical organisation of chromosomes, no genes directly involved with DNA maintenance and repair specific to this stage of the life cycle have yet been isolated.

In general, protein synthesis is reduced in stationary phase cells but some genes, amongst which is *SNZ1,* are induced at a higher rate. The function of the *SNZ1* gene product is not known but it is thought to be involved in cell maintenance during stationary phase (Fuge and Werner-Washburne, 1997). There are 3 *SNZ* genes altogether, each of these has a *SNO* gene upstream. The DNA sequences and relative positions of *SNZ* and *SNO* genes are phylogenetically conserved (Padilla *et al.*, 1998). An analysis of total RNA discovered that *SNZ-SNO* gene pairs are coregulated under various conditions (Padilla *et al.*, 1998). *SNZ2/3* and *SNO2/3* mRNAs are induced prior to the diauxic shift and decrease in abundance during the postdiauxic phase, when *SNZ1* and *SNO1* are induced. This has led to the theory that *SNZ-SNO* associated proteins are part of an ancient response towards nutrient limitation (Padilla *et al.*, 1998).

GRR1, GCS1 and *SED1* are genes associated with cell cycle re-entry. *GRR1* is involved in glucose repression, divalent cation transport and cyclin turnover. Gcs1p is a Zinc finger protein involved in secretion. Several other secretory genes are also involved in stationary phase maintenance, such as, *YPT1* which encodes a GTP binding protein associated with the Golgi apparatus. *GCS1*, *SED1* double mutants do not re-enter the cell cycle or accumulate trehalose and lack mitochondrial DNA (Filipak *et al.*, 1992). *SED1* is the most abundant cell wall

protein in stationary phase cells and is expressed during M phase of the cell cycle.

The *GCS1* gene, previously mentioned, has also demonstrated that stationary phase could be an independent offshoot of the normal mitotic cell cycle. These mutants lose many of the characteristics associated with stationary phase such as resistance to thermo-tolerance and accumulation of glycogen and trehalose and are blocked before start and re-entry into the mitotic cycle in a similar way to cells that have been treated with the mating pheromone **a** factor. This indicates that the cells have not just arrested at a specific point in the cell cycle but need a specific genetic signal to continue proliferating from stationary phase.

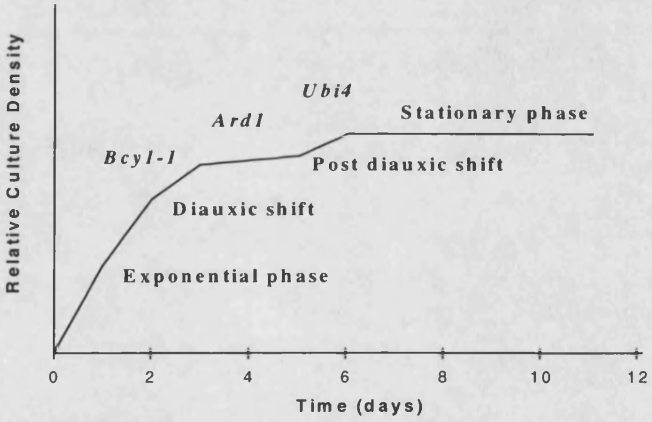


Figure 1.9. The temporal occurrence of mutations which affect entry into stationary phase.

Several mutations cause cells to lose viability at different temporal stages of the growth cycle (Fuge and Werner-Washburne, 1997). *Bcy1-1* is a regulatory subunit of an enzyme involved in signal transduction, protein kinase A. Mutations in the *bcy1-1* gene cause loss of viability at the diauxic shift. *Ard1* encodes a protein that affects *N*-terminal acetylation. These mutants lose viability

after the onset of stationary phase at approximately 5 days. *UBI4* is a gene, associated with heat shock resistance, that encodes polyubiquitin. Mutations in this gene cause loss of viability after approximately 8 days into stationary phase (Fig 1.9).

1.5 Stress responses to environmental extremes.

The responses of yeast cells to various stresses can be used to analyse the effects of different mutations. In *S. cerevisiae* genes related to stress responses are controlled by transcription factors binding to stress response elements (STREs) (Fig.1.10). The core consensus sequences to these elements are AGGGG or CCCCT (Ruis and Schüller, 1995).

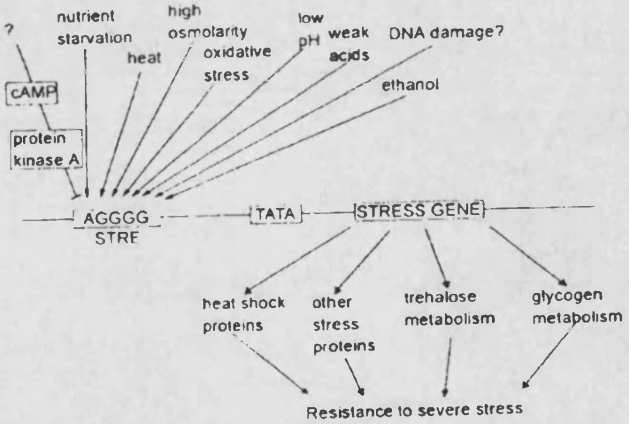


Figure 1.10. The stress gene control mechanism in *S. cerevisiae*. Stress genes are under the regulation of transcription factors binding to stress response elements (STREs) (Ruis and Schüller, 1995).

When starved yeast cells exit stationary phase and return to proliferation in a fermentable media, several metabolic changes take place which are collectively known as the Fermentable Growth Medium-induced (FGM) pathway. Glycogen and trehalose levels decline and trehalases are post-translationally activated. STRE-controlled genes are repressed and cells become more susceptible to stress.

The principal metabolic regulator of the stress control response is protein kinase A activity. When protein kinase A activity is high, trehalose levels and STRE-mediated expression of stress related genes are reduced (Piper, 1993; Siderius and Mager, 1997). Entry into stationary phase and G_1 arrest are accompanied by reduced protein kinase A activity (Siderius and Mager, 1997). It is thought that protein kinase A phosphorylates proteins required for exponential growth and is controlled by input from cAMP, glucose and signalling pathways (Siderius and Mager, 1997).

1.5.1 *Temperature.*

Yeast growth at different temperatures can be used to determine the genetic background of a yeast strain. High temperatures disrupt hydrogen bonding and hydrophobic reactions to denature proteins and nucleic acids. Physiologically extreme temperatures or heat-shock will cause irregular cell wall growth and an increase in cell size. To resist damage, cells will arrest in G_1 of the cell cycle, induces heat shock proteins (hsp's) and accumulate trehalose and MnSOD, an enzyme involved in scavenging free radicals. This physiological state, known as the heat-shock response, is well-characterised in yeast (Piper, 1993; Mager and De Kruijff, 1995). Sudden high temperatures of 40°C or above, activate a heat shock transcription factor (HSF) which induces genes that possess heat shock element (HSE) promoters. The protective function of hsp's are not clearly understood, for, when *S. cerevisiae* is grown in a non-fermenting media, such as acetate, it is thought to acquire heat tolerance without synthesising proteins (Gross and Watson, 1996). However, trehalase may increase temperature

tolerance as strains with trehalase gene deletions become more temperature sensitive when grown in a non-fermentable carbon source (Nwaka *et al.*, 1995).

1.5.2 *Physiological conditions required for Heat Shock Resistance.*

Rapidly growing cells of *S. cerevisiae* are sensitive to heat shock while non-growing stationary phase cells are highly resistant. Resistance also correlates with slow growth and carbon catabolite depression. Slow growing cells show a greater resistance to zymolyase digestion of the cell wall then fast growing cells. Stress resistance is due to a diverse set of physiological adaptations available to cells regardless of cell cycle position (Elliot and Futcher, 1993).

Stationary phase in yeast cells is often compared to G_0 in mammalian cells. Alternatively stationary phase of yeast is compared to an indefinitely prolonged G_1 phase. Several characteristics formerly attributed to G_1 stationary phase cells are exhibited by cells at any stage of the cell cycle, for instance, slowly growing cells have a higher heat shock resistance. Cells grown in ethanol/glycerol are more heat resistant than cells grown in glucose media. There is no significant difference in heat shock resistance in either budded or un-budded cells grown at the same rate. Heat shock resistance has been found to be more dependent on nutritional conditions than cell cycle phase (Elliot and Futcher, 1993; Plesset *et al.*, 1987).

Heat resistance also correlates with carbon catabolite repression. Cells grown on glucose are fully repressed while those grown on glycerol/ethanol are carbon catabolite derepressed. To determine the effects of carbon catabolite repression cells have been grown in YNB medium with glucose and different nitrogen

sources (Elliot and Futcher, 1993). A wide range of growth rates were obtained. Cells grew slower in proline, methionine and tryptophan than in ammonium sulphate with corresponding heat shock resistance. The one exception was growth in leucine were cells grew slower but had increased temperature sensitivity. To further test carbon catabolite derepression, a constitutively derepressed mutant was grown in glucose medium. Although it grew at nearly the same rate as wild type cells it had greater heat shock resistance. When the mutant was grown in glycerol, heat shock resistance increased. This was thought to be due to either a low growth rate or because the residual glucose repression in glycerol medium was removed (Elliot and Futcher, 1993).

1.5.3 *Osmotic stress.*

Yeast cells have no means of actively transporting water, therefore when they are grown in high osmolarity media, such as one with a high salt concentration, they adapt by accumulating glycerol to balance internal osmolarity. In bacteria the equivalent osmoregulatory solutes are amino acids such as betaine, glutamic acid and proline. In yeast the osmoregulatory mechanism is controlled by a MAP (Mitogen Activating Protein) cascade called the HOG (high osmotic glycerol response) pathway (Schuller *et al.*, 1994; Ram *et al.*, 1994). The HOG pathway is initiated by membrane bound receptor proteins which act as osmosensors such as the gene product of *SLN1* (Maeda *et al.*, 1995). In response to low osmolarity the MAP kinase pathway, *SLT2/MPK1,* is activated (Davenport *et al.*, 1995). Both pathways are associated with alterations in cell wall construction (Cid *et al.*, 1995). Cells that are under osmotic stress are also known to accumulate trehalose (Singh and Norton, 1991). Trehalose is thought to be the most effective

saccharide in the stabilisation of biological membranes against osmotic shock (Crowe *et al.*, 1984). Stationary phase cells grown in low water potential are known to increase levels of trehalose and are more osmotolerant than actively dividing cells (Reed *et al.*, 1987). Levels of trehalose also protect cells from osmotic shock when they are transferred to an environment of a higher osmolarity (MacKenzie *et al.*, 1988).

1.5.4 *Oxidative stress.*

Oxidative stress is caused as a consequence of the free radicals produced by the accumulation of reactive oxygen species (ROSs). In yeast cells the majority of ROSs are superoxide anions (O^{2-}), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$). They cause oxidative damage to cellular components, such as, proteins, lipids and DNA. Most of the oxidative damage to DNA is caused by hydroxyl radicals. They can create base lesions by producing forms of thymine glycol, 8-oxoguanine and formamido-pyrimidine. Research has discovered that DNA densely packed into chromatin has a greater resistance to damage than uncondensed DNA (Koshland and Strunnikov, 1996).

ROSs are formed as a consequence of normal metabolic function. Under certain conditions they can be over-generated, for instance, by mitochondrial respiration or by increases in dissolved oxygen tension in growth media. The most abundantly occurring ROSs are superoxides. The superoxide anion is generated predominately by an electron reduction of dioxygen during mitochondrial respiration. In itself the superoxide anion is not highly reactive but it has the ability to react with other molecules to produce more reactive ROSs such as

hydroxyl radicals (Santoro and Thiele, 1997). Yeast has therefore evolved a group of enzymes, called superoxide dismutases (SODs), which can regulate the number of ROSs and reduce oxidative damage. SODs catalyse a reaction that produces hydrogen peroxide as a by-product of a reaction with superoxides (Equation 1.7). Hydrogen peroxide is then converted to water in a reaction catalysed by a family of enzymes known as catalases (Equation 1.8).

Equation 1.7

$$4O^{2-} + 2H^{+} \xrightarrow{\text{SUPEROXIDE DISMUTASE}} O_2 + H_2O_2$$

OXYGEN ION + HYDROGEN ION → OXYGEN + HYDROGEN PEROXIDE

Equation 1.8

$$2H_2O_2 \xrightarrow{\text{CATALASE}} 2H_2O + O_2$$

HYDROGEN PEROXIDE → WATER + OXYGEN

The yeast cell also has several other antioxidant chemicals and enzymes which protect the intracellular environment from ROSs. Glutathione is a thiol compound which scavenges oxygen radicals. Glutathione deficient mutants are hypersensitive to hydrogen peroxide (Izawa *et al.*, 1995) and superoxide anions (Stephen and Jamieson, 1996). The reduction of oxidised glutathione by glutathionine reductase, encoded by *GLR1*, and metallothioneins such as copper-metallothionein also protect cells from damage by oxidants (Grant *et al.*, 1996).

1.5.5 *Metal homeostasis and oxidative stress.*

Generation of ROSs can be brought about by a number of redox reactions in the cell involving metals ions. The two major redox active metal ions that carry out these reactions are Copper ($Cu^{2+/+}$) and Iron ($Fe^{3+/2+}$). These metals are important cofactors of various enzymes in the cell including Cu, Zn superoxide dismutase, cytochrome oxidase and ribonnucleotide reductase (Karlin, 1993; Lippard and

Berg, 1994; Linder and Hazegh-Azam,1996). As a consequence of their reactivity, these ions become toxic at high intracellular concentrations. Mechanisms involved in homeostasis of metal ions are thought to be conserved throughout yeasts and have been extensively studied (Vulpe and Packman, 1995). Copper transport into cells requires a plasma membrane associated Cu^{2+}-Fe^{3+} reductase and two integral membrane proteins, Ctr1p and Ctr3p (Knight *et al.*, 1996). High affinity reduced Fe^{2+} ions are transported into the cell via the plasma membrane-associated Ftr1p and multi-copper ferroxidase associated with Fet3p activity (Askwith *et al.*, 1994). As activity of Fet3p is dependent on levels of Cu^{2+}, disruptions in several of the copper transport genes can affect high affinity Fe^{+} uptake (Dancis *et al.*, 1996).

The yeast vacuole also plays an important role in regulating metal ion homeostasis (Szcypka *et al.*, 1997). Mutations which affect vacuole assembly and vacuolar protein sorting can cause defects in transport of vacuolar enzymes, temperature sensitivity, defective respiration, increased sensitivity to evironmental pH and slow growth on media containing high copper ion concentrations (Eide *et al.*, 1993; Szcypka *et al.*, 1997).

1.5.6 *Iron transport.*

High affinity transport of iron in yeast first requires the reduction of insoluble Fe(III) to the more soluble Fe(II). This is catalysed by the extracellular activity of ferric reductase encoded by *FRE1* and *FRE2*. Ferric reductase activity is also required for copper transport. Transportation of Fe(II) into the cell requires Fet3p, a Cu^{-} dependent glycosylated plasma membrane protein. Fet3p forms a

complex with Ftr1p a plasma membrane protein which allows the binding of copper to Fet3p in the secretory pathway. Iron atoms are thought to bind to Ftr1p via glutamate carboxyl groups. Also found in yeast is a low affinity iron transporter, Fet4p, encoded by *FET4*. The low iron affinity system can also transport Cadmium and Cobalt into the cell. As in the high affinity iron transport, Fet4p requires the activity of ferric reductase provided by Fre1p and Fre2p.

1.5.7 *Heavy metal toxicity.*

Copper transport into *S. cerevisiae* involves *CTR1*. *CTR1* mutants are defective in high affinity copper transport and have a number of copper deficient phenotypes. These include poor growth in copper deficient medium, defective respiration, defective high affinity iron transport and reduced levels of Cu,Zn SOD. Ctr1p is a plasma membrane glycoprotein which has several repeats of a methionine-containing, Copper-binding domain within the amino terminus. On entering yeast cells, copper is distributed to copper requiring proteins, such as, Cu,Zn SOD, cytochrome oxidase and Fet3p.

1.5.8 *The genetics of copper sequestration.*

Protection against elevated levels of copper in *S. cerevisiae* is offered by two genes *CUP1* and *CRS5* which encode isoforms of a metal binding protein called metallothionein (MT). Yeast MTs are small proteins highly enriched in cysteine residues, similar to those found in other higher eukaryotes. Each MT polypeptide binds 6-7 copper atoms *in vitro*. Copper sequestration by MT makes it unavailable for ROS redox reactions. Elevated levels of copper are first recognised by the gene product of *ACE1*, a Cu^{+} metalloregulatory transcription.

The *ACE1* DNA binding domain undergoes a copper-dependent conformational change and can recognise small fluctuations in copper concentration. Ace1p not only activates MT transcription but also activates *SOD1*. It is thought that Cu,Zn SOD requires copper as a catalytic cofactor. Therefore inducing *SOD1* prevents SOD from being overproduced at low copper levels. As copper readily generates harmful reactive oxygen species it would be beneficial for the cell to have additional supplies of Cu,Zn SOD.

1.6 MAP kinase signal pathways and cell integrity.

Mitogen-activated protein kinases (MAPKs) are a diverse group of regulatory proteins that initiate cellular events by phosphorylating other proteins. The MAP kinases, also known as extracellular signal-regulated proteins (ERKs), form the terminal enzymes in a three-kinase cascade. Each member of the cascade activates the next designated enzyme in the series to form a multifunctional, signaling sequence. The signal sequence usually consists of a three protein kinases; a MAPK kinase kinase (MEKK), activates a MAPK kinase (MEK) which in turn activates a MAPK/ERK enzyme. The MEKK enzymes are Ser/Thr protein kinases that activate the MEK enzymes by phosphorylating two serine or threonine residues within a Ser-X-X-X-Ser/Thr motif. The activated MEK enzymes, which are Ser/Thr/Tyr protein kinases, phosphorylate the MAPK/ERK enzymes on Thr and Tyr residues within the Thr-X-Tyr (TXY) consensus sequence. The MAPK superfamily of enzymes are all activated by dual phosphorylation within the TXY consensus sequence present in the activation

loop of the catalytic domain. The central amino acid differs in each member of the MAPK superfamily.

Yeast cells can change morphologically in several ways in response to their environment. Many of the genes responsible for the regulatory pathways that control cell morphology have been cloned and characterised (Cid *et al.*, 1996). The three pathways which have been studied most and have been shown to be directly linked to cell wall synthesis are the pheromone response pathway, PKC1-controlled pathway and the high-osmolarity glycerol pathway (HOG). The activation of these pathways involves a cascade of MAP kinases (Fig. 1.11).

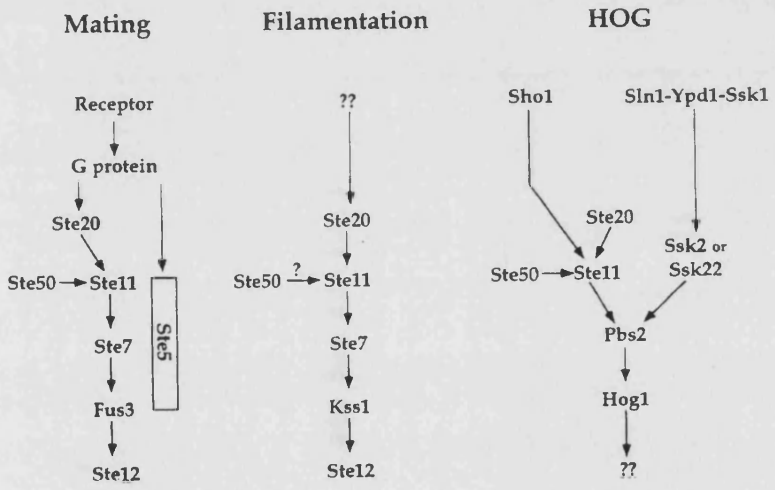


Figure 1.11. Signalling cascades involving MAP kinases: The mating pheromone response pathway, filamentation pathway and the osmosensing pathway (HOG)(Sprague, 1998).

1.6.1 *Pheromone response pathway.*

In *S. cerevisiae*, **a** and α mating factors activate a complex signal transduction pathway which arrest cells at Start and activate the mating process. Yeast pheromones and corresponding receptor proteins interact with a G protein which has GTPase activity. The G protein is composed of three sub-units, α, β and γ.

The α sub-unit of this protein binds to GDP. In response to mating pheromone the GDP is exchanged for GTP causing it to dissociate from the other two subunits of the G protein, β and γ. The β and γ subunits of the G protein then initiate a signalling pathway by activating a protein kinase cascade. The pathway involves the gene product of *CDC42* which modulates polarity of the actin cytoskeleton during budding. This in turn leads to cyclin degradation which inhibits cdc28p, a cyclin dependent kinase, initiating G1 cell cycle arrest and subsequent sexual conjugation.

1.6.2 *PKC1-controlled pathway.*

The *PKC1* pathway is essential for the normal proliferation of actively dividing cells. Several genes have been isolated that are required for the normal functioning of this pathway. These are *PKC1* which encodes a type C protein kinase, *BCK1/SLK1* which encodes a MAP kinase kinase kinase (MEKK), *MKK1* and *MKK2* which encode a MAP kinase kinase (MEK) and *SLT2/MPK1* which encodes a MAP kinase. Strains with a mutation that affects these genes have a lethal cell lysis phenotype at high temperatures (37°C) which can be corrected by an osmotic stabiliser. This phenotype is only observed in proliferating cells and does not affect cells which have entered stationary phase.

1.6.3 *High-osmolarity glycerol pathway.*

S. cerevisiae responds to external increases in osmolarity by synthesising glycerol. This compensates for water loss in the cell caused by osmosis. Osmotically sensitive genes have been isolated by screening for mutants that lose viability when grown at high osmolarity. The genes responsible for regulating the

HOG pathway are *HOG1,* which encodes a MAP kinase, and *PBS2/HOG4,* which encodes a MEK. The pathway is a two component system initiated by a sensor, Sln1p (Maeda *et al.*, 1994). Two signalling pathways converge at the level of the MAPKK Pbs2p. One is triggered by an Slb1p/Ssk1p sensor transmitting the signal by a MAPKKK (Ssk2p/Ssk22p) the other is triggered by Sho1p an alternative sensor to Sln1p. The Sln1p/Ypd1p/Ssk1p/Ssk2p/Pbs2p pathway operates at a low osmolarity in a fast response. While, the Sho1p/Pbs2p operates at a high osmolarity in a slighter longer response. Maeda *et al.*, (1995), propose that the object of the two pathways is to encompass a broad range of osmolarity changes over different time spans.

1.7 Cell wall composition.

Factors that change cell wall assembly in yeast during morphogenesis and the genes involved in its regulation may be of potential interest to the biotechnological, agricultural and pharmaceutical industries. The ability to control cell wall biosynthesis would enhance methods that require increased or decreased cell wall permeability such as the performance of antifungal agents and the production of biosynthetic macromolecules. During conditions of nutrient limitation and stress, fungal cell walls decrease permeability and increase tolerance to environmental extremes. These are all factors that inhibit the functioning of various biological and pharmaceutical control agents. Stationary phase is a mechanism adopted by yeast cells to survive nutrient limitations. On approaching stationary phase yeast cells exhibit several biophysiological modifications and these include changes in the cell wall. Quantifying these

changes would lead to a broader understanding of the genetic control of wall biosynthesis and may allow the development of methods that would control stress resistance and cell wall permeability.

Cell wall analysis of *S. cerevisiae* has been mainly derived from chemical studies. The wall has been divided into four fractions according to solubility in alkaline solutions (Bacon *et al.*, 1969; Cid *et al.*, 1995). Approximately 25% of the cell wall is composed of mannoproteins. The remaining fractions consist of glucan molecules. The greatest fraction, 35% of cell wall weight, is a fibrillar alkali-insoluble acetic-acid insoluble structural component (Manners *et al.*, 1973). Its insolubility in alkali is attributed to a 1,4-β linkage between the terminal reducing end of a chitin chain and the non-reducing end of β1,3-glucan chain (Kollar *et al.*, 1995). When this fraction is treated with chitinase it becomes alkali soluble (Mol and Wessels, 1987). The other two fractions are alkali soluble β1,3-glucan (25% of cell wall by weight) and an alkali-insoluble acetic acid-soluble fraction of β1,6-glucan (5% of cell wall by weight) (Fleet,1991; Fleet and Manners, 1976).

1.7.1 *Yeast cell wall structure.*

The yeast cell wall provides a protective structural barrier between the external and internal environment while retaining the ability to alter integrity throughout stationary phase. At least 90% of the cell wall matrix is dominated by polysaccharides, the remaining 5-10% consists of protein and a small amount of lipids (Fig. 1.12). The polysaccharides exist as polymers of mannose, glucose and

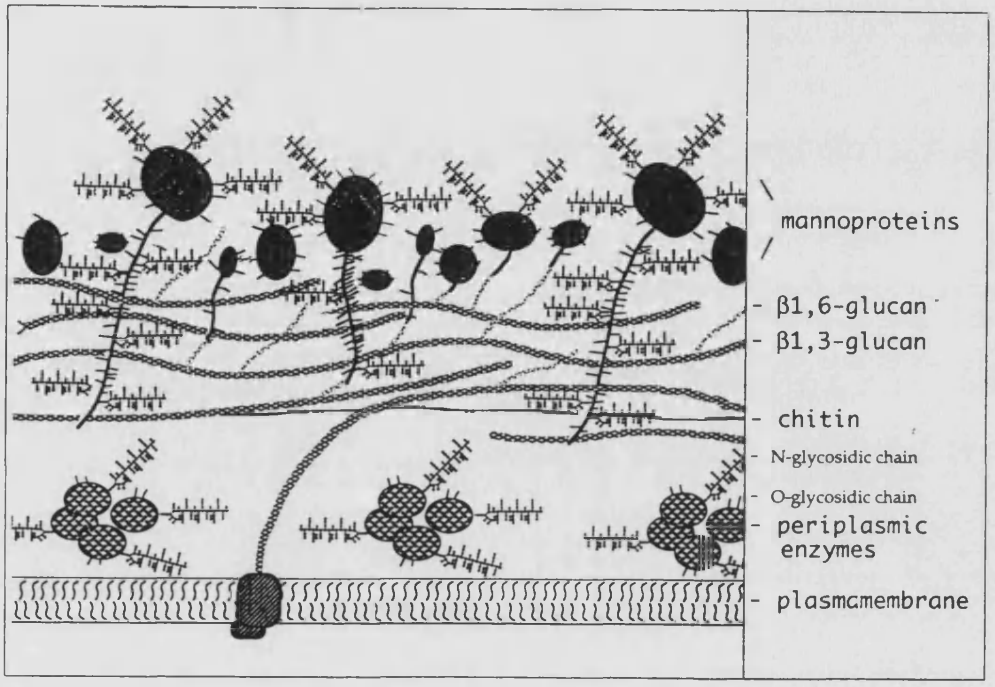


Figure 1.12. Cell wall composition in *S. cerevisiae*. Polysaccharides exit as β1,3-glucan, β1,6-glucan, mannose and chitin. Proteins are covalently linked to mannan to form mannoproteins. Mannoproteins interact with the glucan network. Some mannoproteins are found attached to the plasma membrane but the majority are found in the cell wall (from Schreuder, 1994).

N-acetyl-glucosamine (chitin) (Montijn, 1996). Glucose and mannose polymers are present in about equal proportions. Chitin is found in small quantities, mainly as a result of cell wall repair or in bud scars. The majority of the protein component is covalently linked to mannose to form glycoproteins otherwise known as mannoproteins. Some of these are non-covalently linked to the cell wall and can be extracted with hot sodium dodecyl sulphate but the majority can only be extracted with β-glucanases. All genes encoding glucanase extractable proteins have been found to contain a GPI-anchor attachment signal (Van der Vaart *et al.*, 1996; Caro *et al.*, 1997). The number of cell wall proteins increases in stationary phase cells. Sugar polymerisation occurs by a reaction that uses the nucleotides formed as a consequence of a process involving pyrophosphorylase enzymes (Equations 1.9 and 1.10).

Equation 1.9 Glucose 1-phosphate + UTP → UDP-glucose + PPi

Equation 1.10 Mannose 1-phosphate + GTP → GTP-mannose + PPi

In polysaccharide biosynthesis, transglycosylase enzymes transfer glycosyl moietes to intermediate acceptor molecules such as dolichols. Dolichols are monophosphates of isoprenoid alchohols (C14-18) which act as lipid carriers in the early stages of mannoprotein synthesis.

1.7.2 *β-Glucan*

The synthesis of β1,3-glucan takes place at the cell surface (Fig. 1.13 and 1.14). It requires the sugar donor UDP-glucose and the membrane bound enzyme β1,3-glucan synthase (GS) in the presence of EDTA, GTP and ATP. GS is distributed

in the cell wall in correlation with actin localisation. Rho1p is a GTP binding protein encoded by *RHO1*, that is also localised at sites of growth. It is thought that a prenylated Rho1p exchanges a GDP for GTP, a conformational change then allows it to bind to GS in the cell membrane. This then enables the substrate uridine diphosphate-glucose to bind to a newly created catalytic site and to consequently synthesise β1,3-glucan (Drgonova *et al.*, 1996, Qadota *et al.*, 1996).

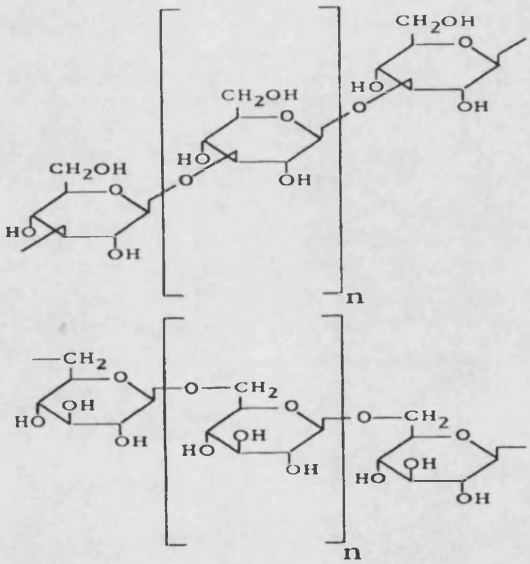


Figure 1.13 Structure of β1,3-glucan (above) and β1,6-glucan (below) (from Schreuder, 1996).

Immunofluorescence evidence shows that actin patches and Rho1p colocalize. A *RHO1*mutation, *V43T*, causes cell cycle arrest at G1, DNA is unreplicated and actin is not redistributed in the cell. Rho1p also interacts with Pkc1p (protein kinase C). Pkc1p mutants display cell integrity defects but Pkc1p is not believed to take place in GS activity (Levin and Bartlett-Heubusch, 1992). *PKC1* mutants show no alteration in GS activity and pkc1p does not restore GS activity in *Rho* mutants. Pkc1p has also not been detected in purified GS complex (Qadota *et al.*, 1996; Drgonova, 1996).

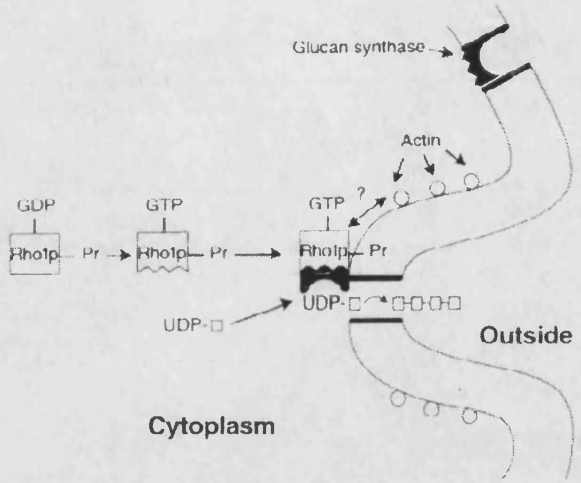


Figure 1.14. Biosynthesis of β1,3-glucan involves the membrane bound enzyme β1,3-glucan synthase (GS). GS catalyses the transfer of glucosyl residues from UDP-glucose to a growing chain of β1,3-glucosyl residues. It is stimulated by a GTP binding protein, Rho1p, which co-localises with actin (Drgonova *et al.*, 1996).

β-Glucans are important in maintaining cell osmotic stability (Ram, 1994). Disrupted cell integrity observed in calcofluor white-hypersensitive mutants may be caused by decreased β1,3-glucan in the cell wall (Ram *et al.*, 1995). Defects in certain genes associated with plasma membrane function e.g., *CWH52* (identical to *GAS1/GSP1*) produce rounded cells with increased and delocalised chitin synthesis and reduced viability in stationary phase. Strains with mutations in some of the *RHO* genes display a cell lysis defect that can be altered by osmotic stabilization (Cid *et al.*, 1995).

1.7.3 *Chitin*

Chitin accounts for only a small percentage of the cell wall. Its role seems to be in repairing structural damage and in strengthening the site of bud initiation. It is mainly deposited in bud scars following cell separation but is found in increasing

quantities in mutants with a structural defect in the cell wall. It forms a ring at the base of the growing bud and then the primary septum in conjunction with the plasma membrane. A secondary septum is then synthesised from β-glucan and mannan. Chitin is also deposited at the subapical end of the shmoo tip when sexual pheromone is added to cells (Lipke *et al.*, 1976).

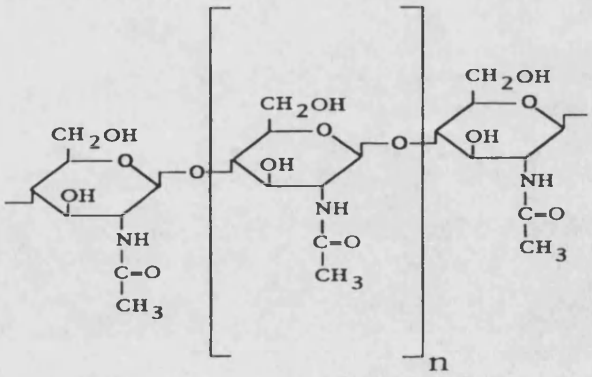


Figure 1.15. Chitin structure (from Schreuder, 1996).

Chitin is a linear polymer of β-1,4-*N*-acetylglucosamine. It is synthesised by the activity of a number of chitin synthases encoded by *CHS* genes. Chitin synthase 1 (CS1) is responsible for the synthesis of the majority (90%) of chitin synthase measured in wild type cells. It is a membrane bound enzyme that catalyses the transfer of *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to an elongating chain of chitin. The gene responsible for the structural component of CS1 is *CHS1*. *CHS1* disruptants grown in minimal media have small refractile buds and frequently lyse at the bud scar leaving a hole in the cell wall at the centre of the bud scar. This is thought to be caused by chitinase activity at the primary septum which facilitates cell separation. It is thought that one of the functions of CH1 is to repair the cell wall after cytokinesis. Chitin synthase II (CSII), encoded by *CHS2*, is also located in the plasma membrane. In strains with reduced CSII activity, the primary septa is replaced by a thick amorphous septum and an

overproduction of chitin synthesis. These factors have led to the theory that CSII is required for normal septum formation (Pammer *et al.*, 1992). The synthesis of the majority of chitin in the vegetative cell is thought to be carried out by Chitin synthase III (CSIII), encoded by several genes including *CHS3, CHS4* and *CHS5* (Cid *et al.*, 1995). Strains with loss of function in any of these three genes have reduced cell wall chitin levels. Chitin rings and chitin deposition in the lateral wall are absent in *chs3* mutants. Only the chitin deposited by CSII in the primary septum is still visible. In *chs4* and *chs5* mutants chitin deposition can still be seen in the lateral cell wall. It is unclear what role these genes serve in CSIII activity but it is thought that they may link chitin synthesis with morphogenesis (Cid *et al.*, 1995).

1.7.4 *Mannoproteins/Cell wall proteins.*

Cell wall proteins (CWPs) or mannoproteins account for 30-50% of the dry weight of the cell wall (Fleet, 1994). There are two main classes of non-detergent extractable cell wall proteins GPI proteins and PIR (protein containing internal repeats) proteins. Both proteins share similar characteristics but, unlike GPI proteins, PIR proteins do not contain a C-terminal GPI addition sequence. All mannoproteins follow the secretory pathway; ER, Golgi complex and secretory vesicles. Within the secretory pathway they are selectively processed at the membrane of the endoplasmic reticulum by the addition of *N*- and *O*-linked side chains or a glycosylphosphatidyl-inositol (GPI) anchor (Caro *et al.*, 1997). When they reach the periplasm they interact with the glucan network to become integrated within the cell wall (Cid *et al.*, 1995; Cabib *et al.*, 1997; Caro *et al.*, 1997). A number of GPI-proteins are found attached to the plasma membrane

but the majority form an intricate part of the cell wall where their function is thought to be in controlling permeability in response to changing growth conditions (Caro *et al.*, 1997). Recent work shows that glucanase extractable proteins are attached by the GPI anchor to the cell wall by a complex series of cross-links involving chitin and β1,6-glucan (Kollár *et al.*, 1997). Mannoproteins are attached to β1,6-glucan by a GPI anchor containing five α–linked mannosyl residues (Fig 1.16). A reducing terminus of chitin chains is attached in β(1,4) and β(1,2) linkages to β1,3- branches of β1,6-glucan. The reducing end of β1,6-glucan is then attached to the non-reduced terminal glucose of β1,3-glucan.

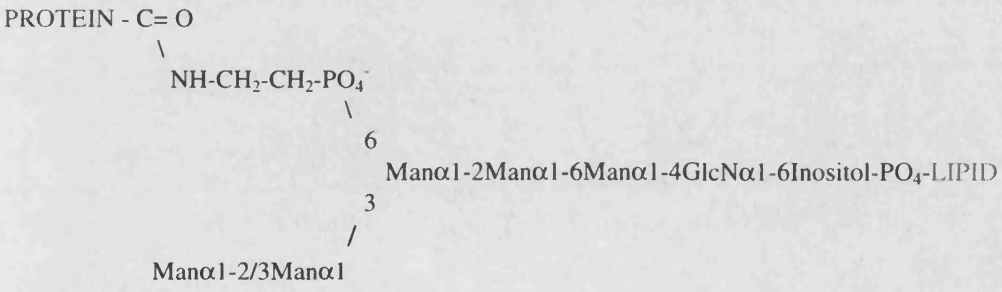


Figure 1.16. Structure of a GPI anchor in yeast (Klis *et al.*, 1998). The C terminal hydrophobic sequence of the protein is exchanged for a pre-assembled GPI anchor in the endoplasmic reticulum by a transamidase.

On approaching stationary phase, cells exhibit increased levels of disulphide bridges and *N*-glycosylation of GPI-anchored mannoproteins (Sanz *et al.*, 1989). In exponentially growing cells GPI-anchored mannoproteins contain the majority of mannose and disulphide bridges. As stationary phase cells contain a six-fold increase in disulphide bridges it was deduced that they must also possess a higher level of GPI-anchored mannoproteins (De Nobel *et al.*, 1990).

1.7.5 *Attachment of GPI anchored cell wall proteins to β-1,6-glucan.*

The process by which β1,6-glucan is incorporated into the cell wall has mainly been studied using mutants that are resistant to *K1* killer toxin. The *K1* killer toxin molecule is a small protein that binds to a β1,6-glucan receptor to form a lethal cation channel across the plasma membrane. The pathway in which β1,6-glucan is constructed has been elucidated by studying a number of *KRE* mutants. *Kre* gene products and their functional homologues have been localised in the ER [*KRE5* (Meaden *et al.*, 1990)], Golgi [*KRE6*, and *SKN1* (Roemer *et al.*, 1994)], the cytoplasm [*KRE1* (Brown *et al.*, 1993)] and the cell surface [*KRE9* (Brown and Bussey,1993)]. Overall, this information implies that synthesis of β1,6-glucan follows the secretory pathway and is then completed at the cell surface but the exact biochemical function of the majority of these gene products is largely unknown (Shahinian *et al.*, 1998). Recently, it has been proposed that β1,6-glucan synthesis takes place at the cell surface while the *KRE* genes are involved in the synthesis of an acceptor structure for later β1,6-glucan addition (Montijn *et al.*, 1998).

1.7.6 *Cell cycle dependence of cell wall protein transcription.*

Mannoproteins vary in size and quantity in relation to population phase. At early exponential phase, mannoproteins range in size from 120-500 kDa with a mean of 200 kDa. In late exponential phase the range varies from 250-350 kDa and the mean is increased to 300 kDa (Zlotnik *et al.*, 1984; Valentin *et al.*, 1987). Recent findings reveal that CWPs appear to have distinct functions and are expressed at different stages of the cell cycle (Caro *et al.*, 1998; Ram *et al.*, 1998). On

investigating the transcription patterns of five CWPs (*CWP1*, *CWP2*, *SED1*, *TIP1* and *TIR1*) it was discovered that *TIP1* was expressed in G_1 phase, *CWP1*, *CWP2* and *TIR1* are expressed in S/G_2 phase and *SED1* in M phase (Caro *et al.*, 1998). In studies using green fluorescent protein as a tag, Cwp1p was localised to small sized yeast buds and the septum whereas Cwp2p was localised all over the cell wall surface, intensifying in the neck region of cells with larger buds (Ram *et al.*, 1998).

1.7.7 *Calcofluor white hypersensitivity.*

To isolate cell wall mutants Ram *et al.* (1994) devised a simple screening method using a selective media containing Calcofluor-White, a fluorescent dye that interferes with cell wall assembly by binding to nascent chains of chitin. It was predicted that strains with a defective cell wall would display increased sensitivity to the dye. After chemically inducing point mutations with ethyl methanesulfonate (EMS) in the yeast strain AR27 (*MATα ura3-52*), colonies (5,000) were transferred to solid media containing calcofluor-white. Strains which showed increased sensitivity to Calcofluor-White were then backcrossed with AR49 (*MATa lys2*). Eventually, following tetrad dissection and another backcross with the wild-type strain, sixty-three single gene cell wall mutants, sensitive to Calcofluor-White, were isolated and placed into 53 complementation groups.

The mutants were then analysed extensively for carbohydrate cell wall composition, response to various chemicals and temperature sensitivity. They were finally categorised and subdivided into three groups according to cell wall

mannose/glucose ratio. It was found that Calcofluor-White sensitivity was not related to the ratio of cell wall carbohydrates as both low mannose (Group 1) and low glucose (Group 3) mutants were amongst the isolates. Three mannose deficient (*mnn*) mutants were discovered in group 1. Four killer resistant (*kre*) mutants most likely affected in β1,6-glucan synthesis were found equally distributed within the three groups. Eleven of the *cwh* mutants were found to be hypersensitive to papulacandin B, which interferes with β1,3-glucan synthesis. Four mutants were temperature sensitive and nine were found to be hypersensitive to caffeine which affects signal transduction in cell wall assembly.

1.7.8 *Cell wall repair mechanism.*

The cell wall of mature cells contains a greater amount of chitin than newly formed daughter cells (Fig 1.17). This is mainly due to chitin deposition which repairs the cell wall after cell separation to form a characteristic budding scar. *S. cerevisae* cells can bud 13 to 30 times depending upon environmental conditions. This results in a cumulative increase of chitin in the cell wall (Kennedy *et al.*, 1994). Recently it has been proposed that chitin also functions as an important component in a cell wall repair mechanism (Kapteyn *et al.*, 1997). Chitin levels are higher in cells that have reduced β1,3-glucan (Kapteyn *et al.*, 1997). Deletion of *FKS1*, which encodes a putative catalytic sub-unit of β1,3-glucan, results in a phenotype with large swollen cells and reduced cell wall β1,3-glucan. In these cells there is a 20 fold increase in chitin linked to CWPs as opposed to the linkage involving β1,3-glucan found in wild type. Similar characteristics occur in

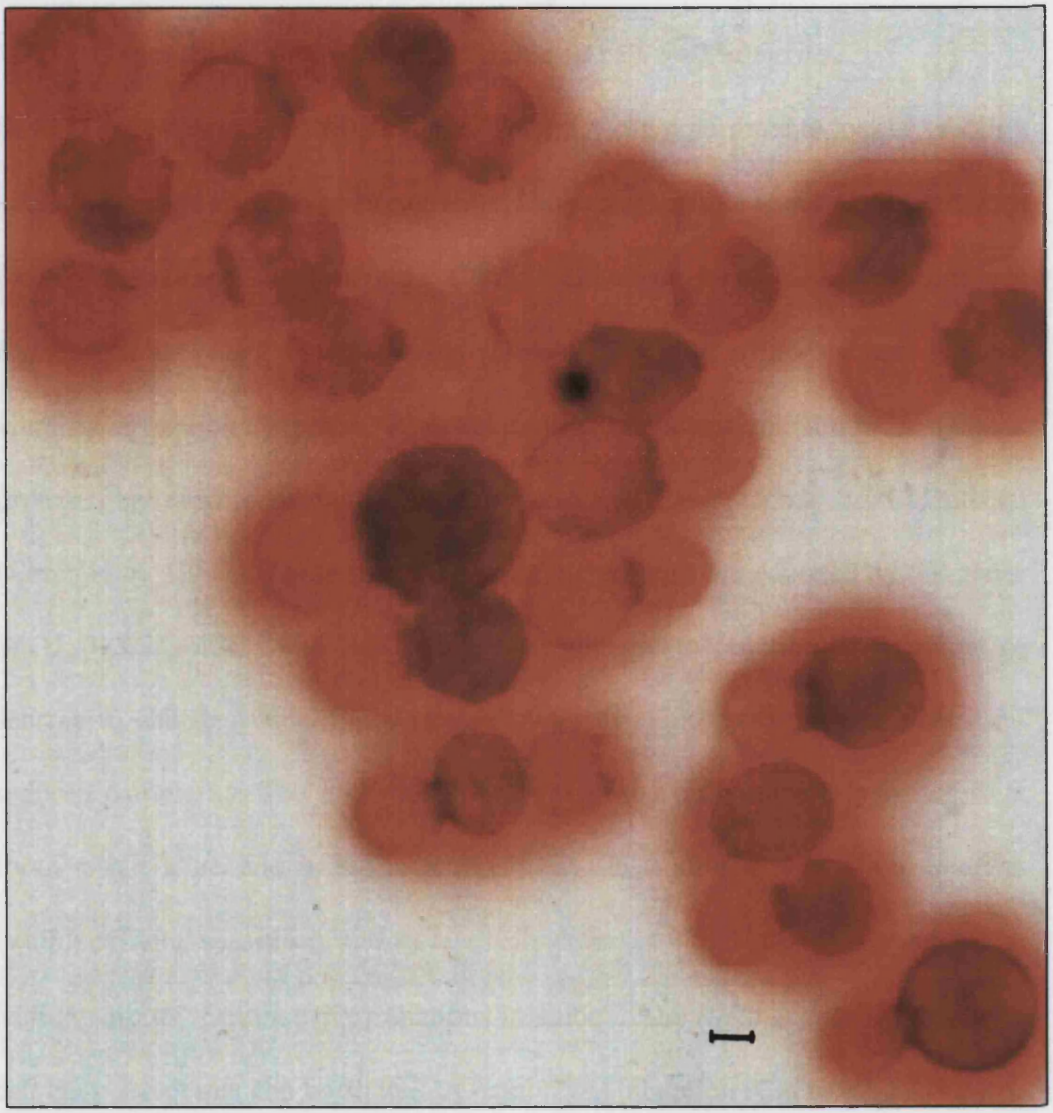


Figure 1.17. Negative image of the fluorescent dye calcofluor white bound to chitin in budding *S. cerevisiae* cells. Chitin deposition is indicated by dark areas. Chitin accumulates in the cell wall of mother cells and is absent in the developing bud. Budding scars are seen as small rings on the mother cell. Scale bar represents 1 μm.

gas1 strains. Both these genotypes also have increased sensitivity to nikkomycin, a chitin synthesis inhibitor (Popolo *et al.*, 1997).

1.7.9 Genes associated with starvation and cell wall integrity.

Several genes associated with cell wall integrity can contribute to loss of viability when yeast cells are starved of nutrients. Defects in certain genes associated with plasma membrane function e.g., *CWH52* (identical to *GAS1/GSP1*) produce rounded cells with increased and delocalised chitin synthesis and reduced viability in stationary phase. A novel family of plasma membrane associated proteins, believed to be environmentally sensitive, have recently been identified (Verna *et al.*, 1997). These integral membrane proteins are encoded by the genes *SLG1*, *WSC2*, *WSC3* and *WSC4*. They are thought to function as receptors or sensors to changes which influence cell wall dynamics such as heat stress and reduced osmolarity. The null mutants of this gene family have a lysis defect at room temperature and heat shock sensitivity. In a similar way *SLG1* mutants exhibit caffeine sensitivity and have a lysis defect at 37 °C that is suppressed by adding sorbitol. Strains with mutations in some of the *RHO* genes also display a cell lysis defect that can be altered by osmotic stabilization (Cid *et al.*, 1995).

1.8 Functional analysis using previously characterised genes.

The advent of Gene Sequencing and the Polymerase Chain Reaction has led to a number of advancements in the functional analysis of characterised genes (Sanger *et al.*, 1977; Mullis and Faloona, 1987). In April 1996, sequencing of the entire 16 chromosomes of the yeast genome was completed (Goffeau *et al.*,

1997). When the yeast genome sequencing project was first initiated only 1,000 genes had been characterised (Mortimer *et al.*, 1992). The recently completed sequence reveals approximately 6,000 protein encoding genes, about 50% of which are of unknown function (Oliver, 1996).

The function of unknown gene products can be predicted by comparing amino acid sequence with proteins from yeast or other organisms. The phenotype caused by the deletion of genes can also be determined. As *S. cerevisiae* has a relatively small genome it is possible to cause deletions in all genes by PCR mediated strategies (Baudin *et al.*, 1993). Gene deletions and over-expression allows phenotypes to be determined by quantitive analysis. Growth and relative concentrations of metabolites can be measured under certain conditions. The sequencing of the genome has also opened the opportunity to analyse the efficient study of RNA expression on a genome-wide scale. A method known as SAGE (Serial Analysis of Gene Expression) has been developed that can identify a Yeast ORF with just 12 nucleotides (Veluilescu *et al.*, 1995; Veluilescu *et al.*, 1996). This method of transcriptome analysis enables the expression levels of a complete set of genes to be measured under specific conditions. Alternative methods to analyse transcriptomes are differential display and hybridization-array technology (Liang and Pardee, 1992; Schena *et al.*, 1996). These newly evolved methods of transcriptome analysis are rapidly replacing traditional Northern approaches. To compliment transcriptome analysis, large scale proteome projects using gel electophoresis are currently being employed to determine protein levels in *S. cerevisiae* under a given set of conditions (Wilkins *et al.*, 1996). These

methods are defining the yeast proteome by 2-dimensional gels using mass spectrometry.

As a result of collaborated projects such as EUROFAN (European functional analysis), which has sequenced the yeast genome and produced the systematic disruption of ~1000 genes. There is now a large amount of information available on databases (e.g., MIPS, *Saccharomyces* Genomic Database (SGD)) to assist with the study of molecular processes in yeast. This study makes use of the information provided by these databases for reference material and in preparing molecular techniques.

Summary

When yeast cells are in stationary phase as a consequence of nutrient limitation they can be subjected to several other stress factors. These can include oxidation, metal ion toxicity, degradation by biological agents and variations in temperature or osmolarity. As a consequence yeast has evolved many cellular processes and mechanisms to resist stress and increase chances of survival. For example, in response to high osmolarity cells protect themselves by synthesising glycerol and accumulating trehalose. The cell wall is a dynamic, protective barrier that is capable of responding to the environment of the cell. By the use of molecular and biochemical techniques, this work aims to assess how the cell wall contributes to stationary phase viability and stress characteristics in *S. cerevisiae*.

Chapter 2

Materials and Methods.

2.1 Strains, media and reagents.

2.1.1 Strains

Strains used in this study are listed below (Table 2.1). AR strains are segregants from a cross between YPH80 (*MATα ade1 ade2 lys2 ura3-52 trp1 leu2-3, 112 his3*) and X2180-1A (*MAT***a**) obtained from the Yeast Genetic Stock Centre (Ram *et al.*, 1994). Calcofluor-white hypersensitive strains were obtained by chemically mutating AR27 *MATα*. These were then crossed with AR49 *MAT***a** (Ram *et al.*, 1994).

Table 2.1. *Saccharomyces cerevisiae* strains used in this study.

Yeast strains:	**Genotype:**	**Reference:**
AM9-10A	*MATα cyr1 bcy1*	Ram *et al.*, 1994
MW301	*MAT***a** *his3 leu ura ard1:: ura3*	"
MW555	*MAT***a** *his3 leu lys2 Δ trp1 ura ubi::leu2*	
AR27	*MATα ura3-52*	Ram *et al.*, 1994
AR49	*MAT***a** *lys2*	"
cwh13-1	*MATα ura3, erd1*	Ram *et al.*, 1994
*cwh*30	*MATα ura3, nrk1*	"
*cwh*41	*MATα ura3, cwh41*	
*cwh*47	*MATα ura3, ptc1*	
*cwh*48	*MATα ura3, kre6*	
*cwh*52-1	*MATα ura3, gas1*	
*cwh*53-1	*MATα ura3, fks1*	
TF27	yEGFP [*URA3*] in AR27	This study
TF13-1	yEGFP [*URA3*] in *cwh13-1*	"
TF30	yEGFP [*URA3*] in *cwh30*	

Table 2.1. (continued).

Yeast strains:	Genotype:	Reference:
TF41	yEGFP [*URA3*] in *cwh41*	This study
TF47	yEGFP [*URA3*] in *cwh47*	"
TF48	yEGFP [*URA3*] in *cwh48*	
SPP27	*sed1Δ::kanMX4* in AR27	This study
SPP13-1	*sed1Δ::kanMX4* in *cwh13-1*	"
SPP30	*sed1Δ::kanMX4* in *cwh30*	
SPP41	*sed1Δ::kanMX4* in *cwh41*	This study
SPP47	*sed1Δ::kanMX4* in *cwh47*	"
SPP48	*sed1Δ::kanMX4* in *cwh48*	
HASED27	*sed1*::HA in SPP27	This study
HASED13	*sed1*::HA in SPP13-1	"
HASED30	*sed1*::HA in SPP30	
HASED41	*sed1*::HA in SPP41	
HASED47	*sed1*::HA in SPP47	
HASED48	*sed1*::HA in SPP48	

2.1.2 Media.

YPD media: Yeast extract 1% (w/v), mycological peptone 2% (w/v), glucose 2% (w/v) or 0.3% (w/v), Bacto-agar 2% (w/v). Sorbitol was used in various concentrations to adjust osmolarity.

SC media: Yeast nitrogen base without amino acids 0.75% (w/v), glucose 2% (w/v) or 0.3% (w/v), Bacto-agar 2% (w/v), amino acids (see Appendix 1).

LB media: Bacto-tryptone, 2% (w/v), Bacto-yeast extract 0.5% (w/v), NaCl 0.05% (w/v), glucose 0.25% (w/v), Bacto-agar 2% (w/v).

2.1.3 Reagents.

TSB: PEG 3350 10% (w/v), DMSO 5% (w/v), 10 mM Mg Cl_2,10 mM $MgSO_4$.

SZB: 1M sorbitol, 100 mM sodium citrate, 60 mM EDTA, 0.5 mg/ml Zymolyase 20T (ICN). 100 mM 2-mercaptoethanol.

SDS-TE: 2% SDS, 0.1M Tris-HCl (pH8), 10 mM EDTA.

DNA manipulation: Bio Taq polymerase (Bioline), T4 DNA ligase (Bioline), Primers (Perkin-Elmer), Restriction enzymes (Amersham Pharmacia Biotech), Calf intestinal alkaline phosphatase (CIP) (Amersham Pharmacia Biotech).

Cell wall degradation: Zymolyase 20T (ICN). 10 mM Tris-HCl (pH7.4).

Trehalose determination: Acid-trehalase (0.05 U/ml) (Sigma), Glucose quantification kit (Sigma), Maleic acid (Sigma).

Drug resistance. Calcofluor-white M2RS (Sigma). $CuSO_4$, Diamide (Sigma).

Cell viability. Phloxine B (Sigma), Methylene blue (Sigma).

Cell wall isolation. Wash buffer: 10mM Tris-HCL (pH 7.8), 1 mM PMSF (Sigma).

Release of cell wall proteins. Extraction buffer: 50 mM Tris-HCL (pH 7.8), 0.1M EDTA, 2% SDS, before use add 2.8µl mercaptoethanol/ml buffer. *Trichoderma* laminarinase (Sigma).

Chitin determination. Glucosamine (Sigma), *p*-dimethylaminobenzaldehyde (Sigma)

PAGE electrophoresis. Running buffer: 0.025 M tris, 0.192 glycine, 0.1% (w/w) SDS, pH 8.3. Sample buffer: 1.0ml dH_2O, 2.5 ml 0.5M Tris/HCl, pH6.8, 2.0 ml glycerol, 4.0 ml 10% SDS and 0.5 ml 0.05% (w/v) bromophenol blue. Stains: Coomassie Blue R-250 (Sigma). Silver nitrate (Sigma).

Western blotting. Transfer buffer: 10% methanol, 24 mM Tris/HCl, 194 mM glycine. Immobilon P Transfer Membrane, PVDF 0.45 pore size (Millipore).

Immunochemistry. Anti–[HA]-peroxidase conjugated mouse monoclonal antibody (Boehringer Mannheim). Diaminobenzidine (Sigma).

2.2 Selection of stationary phase mutants.

Strains were inoculated in Falcon tubes (5 ml) containing 1.5 ml of liquid YPD, with or without an osmotic stabiliser (1M sorbitol) and then incubated at 28°C on a shaker. After a predetermined length of time, drops (5 µl) of culture were placed on solid YPD media (2% (w/v) glucose) or solid SC media (2% (w/v) glucose) containing the vital stain, Phloxine B (0.1 mg/ml). They were then incubated at various temperatures (14°C, 28°C and 37°C) for 24-48 hours. Phloxine B is a viability stain that is excluded by living cells but diffuses into dead cells. Cells that take up the stain are a brighter pink. The main advantage of using phloxine B in this study is that it can be used to screen a large number of mutants simultaneously under identical growth conditions. To estimate cell viability growth index was scored from 0 (no growth) to 3.

2.3 Growth and physiological analysis.

2.3.1 Cell count, budding index and cell viability.

Following sonication (30 seconds), the absorbance of each sample was measured at 600 nm. Cells were counted using a haemocytometer and the percentage of budded cells determined to allow the Budding Index (BI) to be calculated. Serial dilutions made from each sample were spread in 100 µl aliquots on solid YPD

(2% (w/v) glucose) media and incubated at 28-30°C. After 48 hours colonies were counted to determine the number of viable cells from each culture.

2.3.2 Cell size measurements.

***Image splitting microscopy*:** Cell diameter (μm) was determined by microscopically measuring the cell size of twenty individual cells at 100x magnification with an image splitting microscopic attachment (Vickers AEI, Image Splitting Eyepiece). Using this method the mean ± SEM could be determined.

Particle counter: Sonicated samples of cells (50 μl) from individual cultures were also counted and measured by an electrozone particle counter (Elzone 282PC, Particle Data Inc.). Samples were measured in 100 ml of electrolyte (0.9% (w/v) NaCl, 0.1 % (w/v) NaN_3).

2.3.3 Effects of osmolarity on cell growth.

Sorbitol was added to YPD liquid media at various concentrations (0.2 M-1.5 M). Yeast cultures were all started from the same population of yeast cells grown in liquid YPD (2% (w/v) glucose) media to eliminate variations in growth phase. Cell count/size, budding index and cell viability were recorded over a 28 day interval (microscopically and by Elzone particle counter).

2.3.4 Cell wall porosity (Relative DEAE-sensitivity (%)).

Cell wall porosity was measured by assessing relative sensitivity to DEAE (De Nobel *et al.*, (1990)). Under sterile conditions, yeast cells ($4x10^7$/ml) were

washed in distilled water and then shaken (250 rpm) at 28°C for 30 minutes in 4ml of 10mM Tris-HCl (pH 7.4) containing 20 µg DEAE-dextran or 40 µg poly-L-lysine. Following incubation cells were pelleted in a centrifuge for 2 minutes. The supernatant was then filtered (Nikon Millipore HV 0.45 µm, Kogyo KK) and absorbance measured at 260 nm. Measurements were then fitted into *Equation 1.*

Equation 1, Relative porosity $= \frac{(A_{260}\text{DEAE–dextran} - A_{260}\text{buffer}) \times 100}{(A_{260}\text{poly–L–lysine} - A_{260}\text{buffer})}$

2.3.5 Staining cells with calcofluor white.

Cells (~10^7) were pelleted and resuspended in 10 mM HCl/Tris buffer (pH 7.4). Calcofluor white solution was then added to a concentration of 100 µg/ml and incubated (5 min, 20°C). Stained cells were washed twice in distilled water and observed microscopically with a UV filter set.

2.3.6 *Evaluation of cell viability.*

To assess the viability of cells in stationary phase, aliquots of cells from liquid cultures were mixed with an equal volume of methylene blue solution (0.01% (w/v) Methylene blue, 2% (w/v) Na_3 citrate). After 10 minutes incubation at room temperature samples were examined at 100x magnification and counted by haemocytometer to determine the percentage of unstained cells (Sivadon *et al.*, 1997). To assess the ability of cells to reproliferate following stationary phase, viability was also assessed by colony counts on solid YPD (2% (w/v) glucose) from a series of culture dilutions.

2.3.7 *Resistance to calcofluor white, diamide and Cu ions.*

Serial dilutions of cultures were spotted (5 µl) onto solid YPD media containing calcofluor white (1 mg/ml) or solid SC media containing either diamide (1 mM) or $CuSO_4$ (0.75-5 mM).

2.3.8 *Intracellular trehalose determination.*

Trehalose levels have been traditionally measured by extraction with 10% (v/v) trichloroacetic acid then quantified with Anthrone reagent (Lillie and Pringle, 1980). However, Anthrone reagent reacts with different sugars which reduces the specificity of the assay. In this instance, intracellular trehalose levels were measured by an enzymatic assay using an acid-trehalase by a technique described by Kienle *et al.* (1993).

The dry weight of yeast was first determined by drying 5 ml of a 40 ml culture in an oven at 120°C for 2-4 hours. Remaining cells from the 40 ml culture were washed in 0.9% NaCl (w/v) solution, pelleted and resuspended in 40 mM potassium acetate, pH 4.8. The cell suspension was then vortexed with glass beads (500-600 µm) and incubated at 70°C for 5 minutes. Cells were then centrifuged (12,000g, 5min) and the resulting supernatant, diluted 10 times in maleate buffer and used to assess the trehalose content of the culture. Trehalose content was quantified by determining the glucose level of the culture with and without trehalase (0.05 U/ml). To one test-tube was added 20 µl of supernatant and 480 µl of sodium maleate buffer and to another was added 20 µl of supernatant, 460 µl of sodium maleate buffer and 20 µl of trehalase. After incubating for 30 min at 37°C, 1ml of glucose combined solution from a glucose

determination kit was added. This assay determines the quantity of glucose liberated by trehalase hydrolysis. The sample is added to a mixture containing glucose oxidase, peroxidase and o-dianisidine (*Equation 2*). The reaction is taken to completion (30 min, 37°C). Final colour intensity is proportional to glucose concentration.

Equation 2,

$$\text{Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic Acid} + H_2O_2$$

$$H_2O_2 + \underset{\text{(Colorless)}}{\text{O-Dianisidine}} \xrightarrow{\text{Peroxidase}} \underset{\text{(Brown)}}{\text{Oxidised O-Dianisidine}}$$

The samples were incubated for a further 30 min before the absorbance was measured at 470 nm. Absorbance values of trehalose were determined from a calibration curve of trehalose solution (0-40 mg/ml). Intracellular content was determined by fitting values to *Equation 3.*

Equation 3,

$$\text{Trehalose (mg/ml)} = \frac{K\,(\text{Cells } A_{470} - \text{Free Glucose } A_{470})}{\text{Dry weight}}$$

Where K is the gradient of the calibration curve.

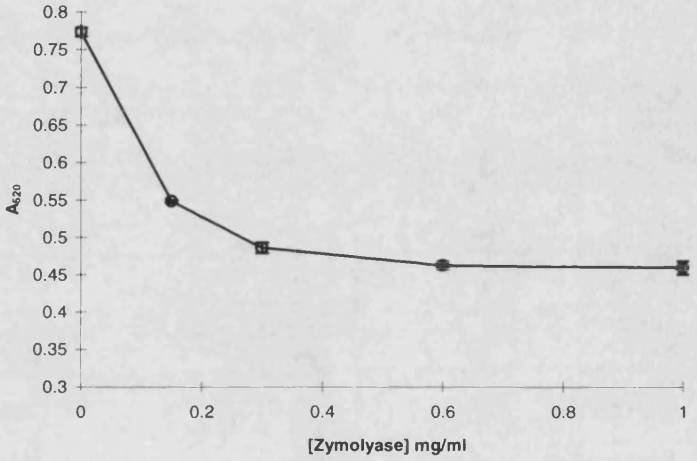
2.4 Cell wall assays.

2.4.1 *Zymolyase resistance.*

Cell walls were degraded with Zymolyase 20T by the technique described by Ram *et al.* (1994). Cells (~$1x10^7$ /ml) were washed and resuspended in 10 mM Tris/HCl, pH7.4. Zymolyase (0.6 mg/ml) was added to 2 ml aliquots of cell suspension. Samples (8x200 µl) were then transferred to a microtitre plate and the absorbance was measured and recorded at 520 nm. After 2 hours incubation, cells were agitated and absorbance was recorded again. Zymolyase is a β1,3-

glucanase enzyme that can be used to detect increased permeability of the cell wall. Mutations which affect mannosylation of cell wall mannoproteins usually have a higher sensitivity to Zymolyase. A calibration curve was constructed to determine the most effective quantity of Zymolyase to use in the assay (Fig. 2.1).

Figure 2.1: Sensitivity of *Saccharomyces cerevisiae* (AR32) exponentially growing cells grown in liquid YPD (0.3% (w/v) glucose) media to Zymolyase 20T. Cells were incubated with Zymolyase for 2 hours at 28 °C. Mean ± SEM, n= 8.



2.3.2 *Cell wall isolation.*

Cell walls were isolated by the method described by Ram *et al.* (1994). Cells were washed several times in 10 ml of ice cold buffer (10 mM Tris-HCl, pH 7.8, 1 mM phenylmethylsulphonyl fluoride (PMSF)) and resuspended in 1 ml of the wash buffer. Glass beads (0.5 mm) were added to just below the meniscus of the suspension and shaken at 4°C until 95% of cells were disrupted (30 mins to 2 h). The cell lysate was separated from the glass beads by repeatedly washing with 1M NaCl. Walls were pelleted (10,000 g, 10 min) and washed two times with 10 ml 1M NaCl and three times with 10 ml wash buffer. Cell walls were stored in buffer at -20°C.

2.4.3 *Release of cell wall proteins.*

Cell wall proteins were analysed by a method described in Klis *et al*, 1998. Wet weight of cell walls was used throughout the experiment. Extraction buffer was added to isolated cell walls (5 µg/mg cell walls) and heated (100°C , 5 min). The suspension was then cooled down to 20°C and cells were washed 5 times with water and once with 0.1 M NaAc (pH 5.5), 1 mM PMSF before resuspending in wash buffer (2 µl/mg of cell walls). A stock solution of laminarinase containing 500 mU/ml was then added to the susupension (0.5 µl/mg cell walls) and left to incubate (37°C, 2 h). Following incubation more laminarinase was added (1 µl/mg cell walls) and incubation was repeated. An equal volume of 2X SDS-PAGE sample buffer was then added to the suspension and heated (100°C, 5 min). The cell walls were pelleted and the supernatant was analysed by SDS-PAGE.

2.4.4 *Protein determination of isolated cell walls.*

Protein assay of isolated cell walls followed a method described by Klis *et al.* (1998). Wet weight of cell walls was used throughout the experiment. Isolated cell walls (30 mg) were suspended in 1 M NaOH (0.1 ml). The suspension was boiled for 10 min and then neutralised with 1 M HCl (0.1 ml). Cells walls were spun for 5 min (10,000 g) and a 50 µl sample was removed from the supernatant. The sample was added to 1 ml of Bradford reagent (Brilliant Blue G in phosphoric acid and methanol). Absorbance at 590 nm was compared to a calibration curve constructed from known amounts of bovine serum albumin. The protein dye complex causes a shift in dye absorption maximum from 465 nm

(brown) to 595 nm (blue). The amount of absorption produced is proportional to protein concentration.

2.4.5 *Determination of chitin content in isolated cell walls.*

Chitin levels in cell walls were measured as described by Dallies *et al.* (1998). Following isolation, cell walls (100 mg (wet wt)) were hydrolysed in 6 M HCL (2 ml) at 100°C overnight in glass tubes sealed with marbles and then placed on a dry block. Tubes were then allowed to cool. The hydrolysate was neutralised with 6 M NaOH and then desiccated by freeze drying (Savant Speed Vac). Following desiccation the samples were resuspended in water (1 ml) and glucosamine was quantified by the following procedure (Popolo *et al.*, 1997; Tracey, 1956). A fresh solution of 0.75 M Na_2CO_3 in 4% (v/v) acetylacetone (0.5 ml) was added to the sample and left to incubate (100°C, 20 min) in glass tubes. After incubation, 3.5 ml of 96% (v/v) ethanol was added followed by 0.5 ml of Ehrlich reagent (1.6g of *p*-dimethylaminobenzaldehyde in 30 ml concentrated HCl and 30 ml ethanol). The absorbance was then measured at 520 nm and compared with a standard curve of 0 to 200 µg of glucosamine.

2.4.6 *Electron Microscopy.*

Cells were prepared for electron microscopy according to Gammie *et al*, (1998) by a method that preserves the ultrastructure of the cell wall. Cells were pelleted and then resuspended in 1 ml of FIX (40 mM potassium phosphate, (pH 7.4), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.2 M sorbitol, 2% (v/v) fresh gluteraldehyde) and incubated for 30 min. After washing three times in 50 mM potassium phosphate,

(pH 7.4), samples were incubated in 4% (w/v) potassium permanganate at 4°C for 5 hours. Cells were washed four times with dH_20 and then re-suspended in 1ml of 0.5-1% (w/v) sodium periodate for 15 min at room temperature to improve infiltration of the resin. Cells were then washed twice in dH_20 and re-suspended in 2% (v/v) filtered uranyl acetate and shaken at 4°C overnight. To dehydrate the cells they were washed in 50% (v/v) ethanol and 70% (v/v) ethanol for 5 min two times, 95% (v/v) ethanol for 5 min, and 100% (v/v) ethanol for 5 min three times. Finally samples were embedded in LR White resin and cut into sections. Sections were then observed and recorded by transmission electron microscopy at an acceleration voltage of 80.0 KV.

2.5 Molecular techniques.

2.5.1 *DNA extraction and purification.*

Yeast genomic DNA was isolated and prepared as described by Strathern and Higgins (1991). Washed and pelleted cells from 10 ml cultures were re-suspended in SZB (0.15 ml) and incubated at 37°C for 1-2 hours. SDS-TE (0.15 ml) solution was added to resulting spheroplasts which were incubated for a further 10 min at 65°C. Cells were then incubated on ice with 5 M potassium acetate solution (0.15 ml) for 30-45 min and centrifuged (9,000 g, 15 min) at 4°C. The pellet was discarded and to the supernatant was added 5 M ammonium acetate (0.2 ml) and pre-chilled 2-propanol (1 ml). This was incubated overnight at -20°C then centrifuged (12,000 g, 5 min). The pellet was rinsed in 80% (v/v) ethanol, air dried and re-dissolved in water containing 2 µl of 10 mg/ml RNase. Following 10 min incubation at 37°C, the DNA was extracted with phenol (200

ml) then centrifuged (12,000 g, 2 min). The supernatant (aqueous phase) was precipitated with ethanol then centrifuged (12,000 g, 2 min). The resulting DNA pellet was re-dissolved in water (50 µl) and stored at -20°C. For DNA sequencing, the PCR product was purified by a Sephaglas purification kit according to manufacturer's instructions (Sephaglas band prep kit, Pharmacia Biotech).

2.5.2 *Molecular cloning.*

Plasmids were cloned by transforming into *Escherichia coli* strain DH5α by a procedure described by Sambrook *et al* (1989). Cells were made competent for transformation by a simple technique using DMSO (Chung and Miller, 1988). Early log phase cells (0.3-0.6 A_{600}), grown in LB media at 37°C, were made competent by re-suspending pelleted cells in 1/10 original volume of TSB (see section 2.1.3). Cells were incubated on ice for 30 min before transforming with DNA. Approximately 1 ng of DNA was added to 50 µl of competent cells. They were left on ice for 30 minutes and then heat shocked at 42°C for exactly 90 seconds. Cells were then allowed to recover by incubating in 200 µl liquid LB media (45 min, 37°C). Aliquots (200 µl) of cells were transferred to solid LB media containing ampicillin (50 mg/L). Resulting transformants were cultured in liquid LB media with ampicillin (50 mg/L). Plasmid DNA was isolated from transformants by ion-exchange column chromatography using a Wizard Plus SV minipreps DNA purification system.

2.5.3 *Yeast transformation.*

Transformation in *S. cerivisiae* was as previously described by Gietz and Schiestl (1995). Cells were grown in YPD to early exponential phase, and then harvested by centrifugation (5,000 g, 5 min). They were washed twice in water and then resuspended in 100 mM LiAc. A transformation mix containing 50% (w/v) PEG (3350) (240 µl), 1M LiAc (36 µl), single-stranded carrier DNA (2 mg/ml)(25 µl) and 10 µg transforming DNA in dH_20 (50 µl) was added to pelleted cells. The mix was incubated at 30°C for 30 min, and heat shocked at 42°C for 25-30 min. Cells were then plated on to selective media and incubated at 30°C for 3 days.

2.5.4 *Synthesis of disruption cassettes with small flanking homology PCR (SFH-PCR).*

Gene disruption was carried out by a one-step gene deletion using the PCR product of *kanMX4* by a procedure first described by Wach *et al.*, (1994). *S. cerevisiae* acquires resistance to the drug geneticin (G418) when transformed with *kanMX*.

A DNA fragment containing the kanamycin resistant module (*kanMX4*) flanked by primers from the target gene was first amplified from *Not*I digested pFA6-kanMX4. Oligonucleotide primers encode a sequence of DNA from 5′ to 3′, primer S1 (or S2) has 40 nucleotides homologous to the 5′-site (or 3′-site) of the *S. cerevisiae* genomic target locus followed by 18-19 nucleotides of *kanMX4* DNA (Table 2.2). The *kanMX* module was amplified using these primers in a 50 µl PCR mix [10 µl (x10) NH_4 buffer, 0.2 mM dNTPs, 3 µl 50mM $MgCl_2$, 1µM Primers, 0.5 µg *Not*I digested pFA6-kanMX4, 0.5 µl 25U DNA

Bio-X-act polymerase (Bioline), to 50 µl with deionised dH_2O]. The PCR amplified product (10 µl, ~1-5 µg DNA) was transformed into haploid strains by the LiAc method described previously (see section 2.5.3). Transformants were selected by incubating for three days at 28°C on solid YPD media containing geneticin (G418) 200-250 mg/l. The *kanMX4* module in transformants was located by PCR analysis and confirmed by gene sequencing (Appendix 3).

2.5.5 *Endonuclease digestion and DNA ligation.*

***Restriction endonuclease digestion*:** DNA (~1µg) was incubated with the appropriate restriction enzyme(s) (1 µl of 15U/ml) and compatible buffer solution made up to 30 µl with dH_2O (1-2 h, 37°C). The reaction was stopped by incubating at 65°C for 15 minutes. DNA was then purified by agarose gel electrophoresis and a Sephaglas purification kit according to manufacturers instructions (Sephaglas band prep kit, Pharmacia Biotech).

***Dephosphorylation of linearized vector DNA*:** Dephosphorylation is mainly used when a plasmid contains identical termini. It removes the 5'-phosphatase groups from both termini to prevent recircularisation. Plasmid DNA was first digested with appropriate restriction endonucleases then purified. Calf intestinal alkaline phosphatase (CIP) was used in dephosphorylation reactions. DNA was incubated with 10 µl of (10x) CIP dephosphorylation buffer and 0.01 U/pmol CIP (30 min, 37°C). The CIP was inactivated by heating to 65°C for 1 hour in the presence of EDTA (pH 8.0). DNA was then purified and stored at –20°C.

***Ligation reactions*:** Ligation was performed using the procedure outlined by Sambrook *et al.* (1989). Dephosphorylated vector DNA with two different sticky-ended termini was used to reduce deconcatermerisation and recircularisation.

The following test reactions were set up;

A: 200 ng dephosphorylated vector DNA

B: 200 ng dephosphorylated vector DNA + 200 ng DNA insert

C: 200 ng untreated vector DNA + 200 ng DNA insert

To each sample was added; 1 µl (10x) ligation buffer, 1 µl 10 mM ATP, 10 µl dH_2O. To each reaction mix was added 0.5 Weiss unit of bacteriophage T4 DNA. The reactions were then incubated overnight at 12°C. Competent *E.coli* were transformed with 1 µl of each ligation reaction and a control of closed circular plasmid (see 5.5.1). Positive transformants were tested for DNA insertion by performing a PCR on lysed *E. coli* cells .

2.5.6 *Construction of Sed1-GFP fusion proteins.*

A GFP fusion gene was constructed from a series of GFP-fusion vectors with a gene that has enhanced fluorescence properties, yEGFP (Cormack *et al.,* 1997). The *SED1* region was amplified from genomic DNA extracted from AR27 with primers containing terminal *Eco*RI/*Hin*dIII restriction sites (Table 5.2). The PCR product was confirmed as *SED1* by digesting with *Kpn*I. The *Eco*RI/*Hin*dIII digested DNA fragment containing *SED1* was inserted into *Eco*RI/*Hin*dIII digested pUG36 (yEGFP3, *N*-FUS, URA3 (Figure 2.2)) and ligated to give a sequence that would eventually encode a GFP protein fused to the *N*-terminus end of Sed1p.

The GPI anchor required for cell wall attachment is located at the C-terminus end of Sed1p. The ligated plasmid was transformed and cloned into *E. coli*. Insertion of the DNA fragment into pUG36 was confirmed by PCR analysis.

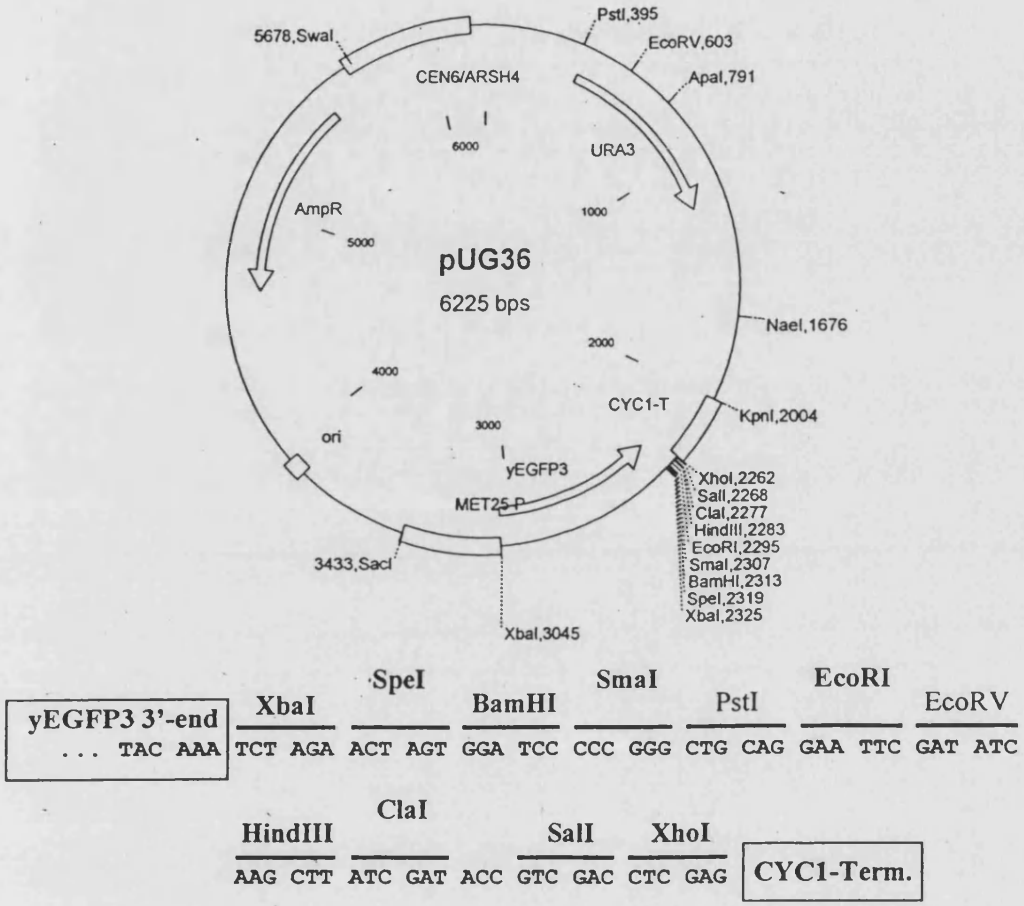


Figure 2.2. yEGFP3-*N*-FUS vector, pUG36.

2.5.7 Epitope tagging of Sed1p.

An HA-epitope tag was inserted into *SED1* using a site-directed mutagenesis PCR procedure (Ho *et al.*, 1989, Horton *et al.*, 1989). Using this technique two PCR products were generated with overlapping complementary ends. The two PCR products are then fused together in one reaction to construct a DNA fragment with an inserted sequence. The method used to HA-epitope tag *SED1* is described by Ram *et al.* (1998) and Shimoi *et al.* (1998). A 3.3 kb fragment of

DNA containing *SED1*, promoter regions and terminal *Eco*RI/*Bam*HI restriction sites was first amplified by PCR from AR27 genomic DNA. The hydrophobic regions of Sed1p are removed during modification processes in the ER. Therefore, an HA-epitope sequence flanked by *Asc*I/*Not*I was inserted after the

Table 2.2: Oligonucleotide primers used in this study. Direction of sequence 5'→ 3'.

***SED1* gene disruption.**

S1

5' TGTCCTATTATCTGCCGGTTTAGCCTCGACTACTTTGGCC**CGTACGCTGCAGGTCGAC** 3'

S2

5' AACATAGCAACACCAGCCAAACCTAAAGCACCTGGAACGA**ATCGATGAATTCGAGCTCG** 3'

Kanamycin primers in bold.

***KanMX4* location.**

A1 5' TACGAAAGAGGAGAAGGG 3'

KAN 5' CAGTTCTCACATCACATCC 3'

***SED1* gene.**

SEDf 5' **GGAATTC**ATGAAATTATCAACTGTCCTATTATCTGCCGG 3'

*Eco*RI site in bold.

SEDr 5' **CCCAAGCTT**TTATAAGAATAACATAGCAACACCAGCCAAACC 3'

*Hin*dIII site in bold.

Fragment containing *SED1* gene and *Eco*RI/*Bam*HI restriction sites.

Frag*Eco*RIf 5' AAC**GAATTC**GAATCCTTGGAAGAATA 3'

*Eco*RI site in bold.

Frag*Bam*HIr 5' GCT**GGATCC**TCATAGCTTATCAAAG 3'

*Bam*HI site in bold.

HA epitope insertion into *SED1*.

HAfSED1

5'TACCCATACGACGTCCCAGACTACGCT**GGCGCGCC**CAATTTTCCAACAGTACATCTGCT 3'

HA epitope sequence underlined. *Asc*I restriction site in bold.

HArSED1

5'AGCGTAGTCTGGGACGTCGTATGGGTA**GCGGCCGC**GGCCAAAGTAGTCGAGGCTAAACC 3'

HA epitope sequence underlined. *Not*I restriction site in bold.

hydrophobic region at the *N* terminal end of *SED1* by PCR hybridisation using the primers indicated in Table 2.2. This was achieved by generating two gene products with the appropriate forward and reverse primers (**Frag*Eco*RIf+HArSED1** and **HAfSED1+ Frag*Bam*HIr**) using either the *Eco*R1/*Bam*H1 DNA fragment or genomic DNA as a template. The two resulting PCR products were then used as a template in a reaction primed by the two terminal oligonucleotides (**Frag*Eco*RIf +Frag*Bam*HIr**).

2.5.8 PCR analysis of transformed cells.

To confirm the transformation of vector DNA into *E. coli* or *S. cerevisiae*, cells from a single colony were transferred with a pipette tip to a PCR tube. The cells were then lysed by heating in a microwave for 1 minute. A 50 µl PCR mix (Appendix 1) was then added to the lysed cells and PCR was performed under the following conditions. Initial denaturation (120 sec, 94°C) followed by 30 cycles of denaturation (30 sec, 94°C), annealing (30 sec, 47°C) and elongation (90 sec, 72°C). Then a final elongation (120 sec, 72°C) and cooled to 2°C. The PCR product was then analysed by electrophoresis.

2.6 Immunochemistry and SDS-PAGE.

2.6.1 *Electrophoresis.*

Appropriate gradient and separating acrylamide gels were prepared (Appendix 1) and placed in a protein electrophoresis tank with running buffer. Samples (20 µl), that had been heated (100°C, 5 min) in loading buffer and centrifuged (12,000 g,

2 min), were injected into wells with a pipette. Gels were then run at 10 V/cm for approximately 2 hours.

2.6.2 *Staining techniques.*

Coomassie Blue staining. After running, acrylamide gels were covered in Coomassie blue solution (0.5 g Coomassie Blue, 800 ml methanol, 140 ml acetic acid made up to 2.0 L with dH_2O) and left to incubate for 2 hours with constant shaking. They were then washed in dH_20 and placed in destain (7.5% (v/v) acetic acid) for five hours.

Diamine silver staining. A silver staining technique selectively sensitive to glycoproteins was used (Merril, 1990). After running, acrylamide gels were washed for 5 mins in dH_2O and then placed in a solution containing 5% (v/v) ethanol, 5% (v/v) acetic acid and dH_2O for 3 hours. After washing for 5 min in dH_2O they were soaked for 30 min in 10% (v/v) gluteraldehyde. Gluteraldehyde solution was removed by five 30 min washes in dH_2O. Gluteraldehyde treated gels were soaked for 10 min in ammoniacal silver nitrate solution. Ammoniacal silver nitrate solution was prepared by slowly adding 30 ml of 1.2 M silver nitrate solution to a solution containing 10 ml of concentrated ammonium hydroxide and 1.5 ml of 10 M sodium hydroxide in 160 ml dH_2O. This solution was then adjusted to 750 ml with dH_2O. Gels were removed and washed three times (x5 min) in dH_2O. Gels were developed in a solution containing 0.1 g of citric acid and 1ml of formaldehyde (37% commercial formaldehyde) per litre dH_2O. When the image was developed (approx. 3 min) the reaction was stopped by placing the gels in a solution containing 5% (v/v) acetic acid. The acetic acid was then removed by washing the gels in 10% (v/v) ethanol solution.

2.6.3 *Western blot procedure.*

For each gel, 2 pieces of Whatman 3 MM filter paper and 1 piece of PVDF membrane were soaked in transfer buffer for 5 min. A foam pad was placed on the black half of a transfer cassette then on the top of this was placed a piece of soaked filter paper and the acrylamide gel. The soaked PVDF membrane was placed on top of the gel followed by another piece of soaked filter paper and a foam pad. The transfer cassette was folded over and placed in the transfer apparatus in transfer buffer. The apparatus was run at 10 V/cm for 1.5 hours in a cold room. To confirm that the protein has transferred to the PVDF membrane, gels were stained with Ponceau S in a Petri dish. Proteins appear as a red band. The stain was then removed from the PVDF membrane by rinsing with dH_2O.

2.6.4 *Immunochemistry.*

Following electrophoresis and western blotting, HA-tagged proteins were detected by the following procedure. The PVDF membrane was first incubated at room temperature for 1h in a blocking agent (non-fat dry milk (5% w/v) dissolved in PBS). The blocked membrane was then added to a 1000:1 solution of anti-[HA]-peroxidase conjugate reagent (10 µl of anti-[HA]-peroxidase to 10 ml PBS). This was incubated for 1 hour at room temperature with gentle rotation and then washed four times (10 min/wash) with PBS, 0.1% Tween 20. Peroxidase activity was detected by adding the substrate solution (diaminobenzidine (0.1% in 0.1 M Tris/HCl, pH7.2)) mixed with 0.02% hydrogen peroxide (in dH_2O from a 30% stock). This was left to develop for 5 minutes, washed twice in distilled water and then stored in the dark.

Chapter 3

Influence of cell wall composition on viability in stationary phase.

3.0 Introduction

The influence of cell wall composition in stationary phase was determined by screening strains of *Saccharomyces cerevisiae* that are Calcofluor White hypersensitive (*cwh*). Calcofluor-White is a cell surface-specific, fluorescent dye that binds to nascent chains of chitin through hydrogen bonding and dipole (van der Waals) interactions. Cells with certain defects in cell wall assembly are hypersensitive to the drug. The *cwh* strains, screened in this study, were from a library created by Ram *et al.*, (1994). Using a novel approach to select for defective cell wall assembly, Ram *et al.* (1994) isolated 63 monogenic mutants from 5,000 colonies after chemical mutagenesis with ethylmethane sulphonate (EMS). EMS induces single base substitutions in a two step process. The *O*-6 position of guanine is first ethylated; then during DNA replication the ethylated nucleotide pairs with thymine.

After inducing point mutations in the yeast strain AR27 (*MAT*α *ura3-52*), colonies (5,000) were transferred to solid media containing Calcofluor-White. Strains with increased sensitivity to Calcofluor-White were then back-crossed to AR49 (*MAT***a** *lys2*). Resultant mutants sensitive to Calcofluor-White were classified into 53 complementation groups and then further categorised into sub groups according to cell wall mannose/glucose ratio and by other phenotypic tests involving drugs that are known to affect cell surface synthesis

Table 3.0.1: Cell wall composition and phenotypic analysis of calcofluor white hypersensitive (*cwh*) strains as described by Ram *et al.*, (1994).

		Cell wall composition (%)*			Man/Glc	
	Strain	**GN**	**Glc**	**Man**	**(mean ± SE)****	**Additional phenotypes**
Group 1	*cwh1*	4.1	83.3	12.6	0.16 ± 0.01	Zymolyase-hs
	cwh2-1	3.8	78.7	17.5	0.23 ± 0.02	Vanadate-resistant, Zymolyase-hs
	cwh3	2.5	78.3	19.2	0.25 ± 0.05	Papulacandin B-hs, Zymolyase-hs
	cwh4	2.8	71.5	25.7	0.37 ± 0.09	Ts lytic, Zymolyase-hs
	cwh5	2.7	69.5	27.8	0.40 ± 0.01	Zymolyase-hs
	cwh6	3.4	66.2	30.4	0.46 ± 0.04	Ts lytic, Zyinolyase-hs
	cwh7	1.1	67.6	31.3	0.47 ± 0.01	Ts lytic
	cwh8	2.9	66.2	30.9	0.47 ± 0.01	Zymolyase-hs
	cwh9	2.2	66.7	31.1	0.47 ± 0.01	Zymolyase-hs
	cwh10	2.5	65.4	32.1	0.50 ± 0.04	Zymolyase-hs, caffeine-hs
	cwhl1	2.7	63.4	33.9	0.54 ± 0.02	Zymolyase-hs
	cwh12	2.4	62.3	35.3	0.57 ± 0.02	Papulacandin B-hs
	cwh13-1	3.6	59.5	36.9	0.62 ± 0.03	Killer-resistant, Zymolyase-hs
	cwhl4	1.4	59.9	38.7	0.65 ± 0.01	Caffeine-hs
	cwhl5	1.5	57.7	40.8	0.71 ± 0.03	
	cwhl6	3.0	56.2	40.8	0.73 ± 0.03	
	cwh17	1.3	57.4	41.3	0.75 ± 0.03	Caffeine-hs
	cwh18	0.9	56.4	42.7	0.76 ± 0.02	Papulacandin B-hs, Rho-
	cwhl9	1.0	56.1	42.9	0.77 ± 0.03	Caffeine-hs
	cwh20	1.2	55.6	43.2	0.78 ± 0.03	Papulacandin B-hs, Zymolyase-hs
	cwh21	1.2	55.6	43.2	0.78 ± 0.02	
	cwh22	1.6	54.8	43.6	0.80 ± 0.03	Papulacandin B-hs, caffeine-hs
Group 2	*cwh23*	1.8	53.5	44.7	0.84 ± 0.03	
	cwh24	1.2	53.4	45.4	0.85 ± 0.04	
	cwh25	1.3	53.2	45.5	0.86 ± 0.04	
	cwh26	1.6	51.6	46.8	0.91 ± 0.01	Papulacadin B-hs, Zymolyase-hs, caffeine-hs
	cwh27	1.2	51.6	47.2	0.92 ± 0.06	Rho-
	cwh28	1.2	51.3	47.5	0.93 ± 0.05	
	cwh29	1.3	50.0	48.7	0.93 ± 0.02	
	AR27	**0.9**	**49.8**	**49.3**	**0.94 ± 0.04**	**Parental strain.**
	cwh30	1.4	50.7	48.9	0.95 ± 0.01	Killer-resistant
	cwh31	1.2	50.5	48.3	0.96 ± 0.08	Zymolyase-hs
	AR49	**1.3**	**51.3**	**47.4**	**0.97 ± 0.05**	**Parental strain.**
	cwh32	0.8	50.0	49.2	0.99 ± 0.03	Papulacandin B-hs, caffeine-hs
	cwh33	1.0	49.9	49.1	0.99 ± 0.02	
	cwh34	1.2	49.3	49.5	1.00 ± 0.01	Zymolyase-hs
	cwh35	1.4	49.0	50.6	1.01 ± 0.01	
	cwh36	1.2	48.2	50.6	1.05 ± 0.07	Papulacandin B-hs
	cwh37	0.7	47.4	51.9	1.10 ± 0.08	
	cwh38	1.2	46.7	52.1	1.12 ± 0.14	
	cwh39	2.0	45.8	51.2	1.14 ± 0.01	
	cwh40	1.1	45.4	53.5	1.18 ± 0.05	Zymolyase-hs
Group 3	*cwh41*	1.2	45.0	53.8	1.20 ± 0.01	Killer-resistant
	cwh42	0.7	44.8	54.5	1.22 ± 0.01	Zymolyase-hs
	cwh43-1	1.3	44.3	54.4	1.22 ± 0.06	
	cwh44-1	1.5	43.9	54.6	1.25 ± 0.02	Zymolyase-hs
	cwh45	1.7	42.4	55.9	1.32 ± 0.02	
	cwh46-1	1.6	41.6	56.8	1.37 ± 0.04	Papulacandin B-hs, Zymolyase-hs, caffeine-hs, Rho
	cwh47	1.1	41.3	57.4	1.39 ± 0.02	Killer-resistant
	cwh48	2.2	38.7	59.1	1.54 ± 0.13	Killer-resistant
	cwh49	1.5	38.4	60.1	1.57 ± 0.10	
	cwh50	1.7	38.1	60.2	1.58 ± 0.05	Ts lytic
	cwh51	1.0	34.5	64.5	1.87 ± 0.09	Papulacandin B-hs, caffeine-hs
	cwh52-1	3.9	30.7	65.4	2.13 ± 0.10	
	cwh53-1	3.3	19.7	77.0	3.92 ± 0.03	Papulacandin B-hs

* GN, *N*-acetylglucosamine; Glc, glucose; Man, mannose.

** mean ± standard error. hs, hypersensitive. In all cases two cell wall preparations were analysed obtained from separate cell cultures.

and regulation (Table 3.0.1). They were then further categorised and subdivided into three groups according to cell wall composition. Group 1 contained mutants with a low mannose/glucose ratio (0.16-0.80), group 2 contained mutants with an approximately wild-type ratio of mannose/glucose (0.84-1.18) and group 3 contained mutants with a high mannose/glucose ratio (1.20-3.92).

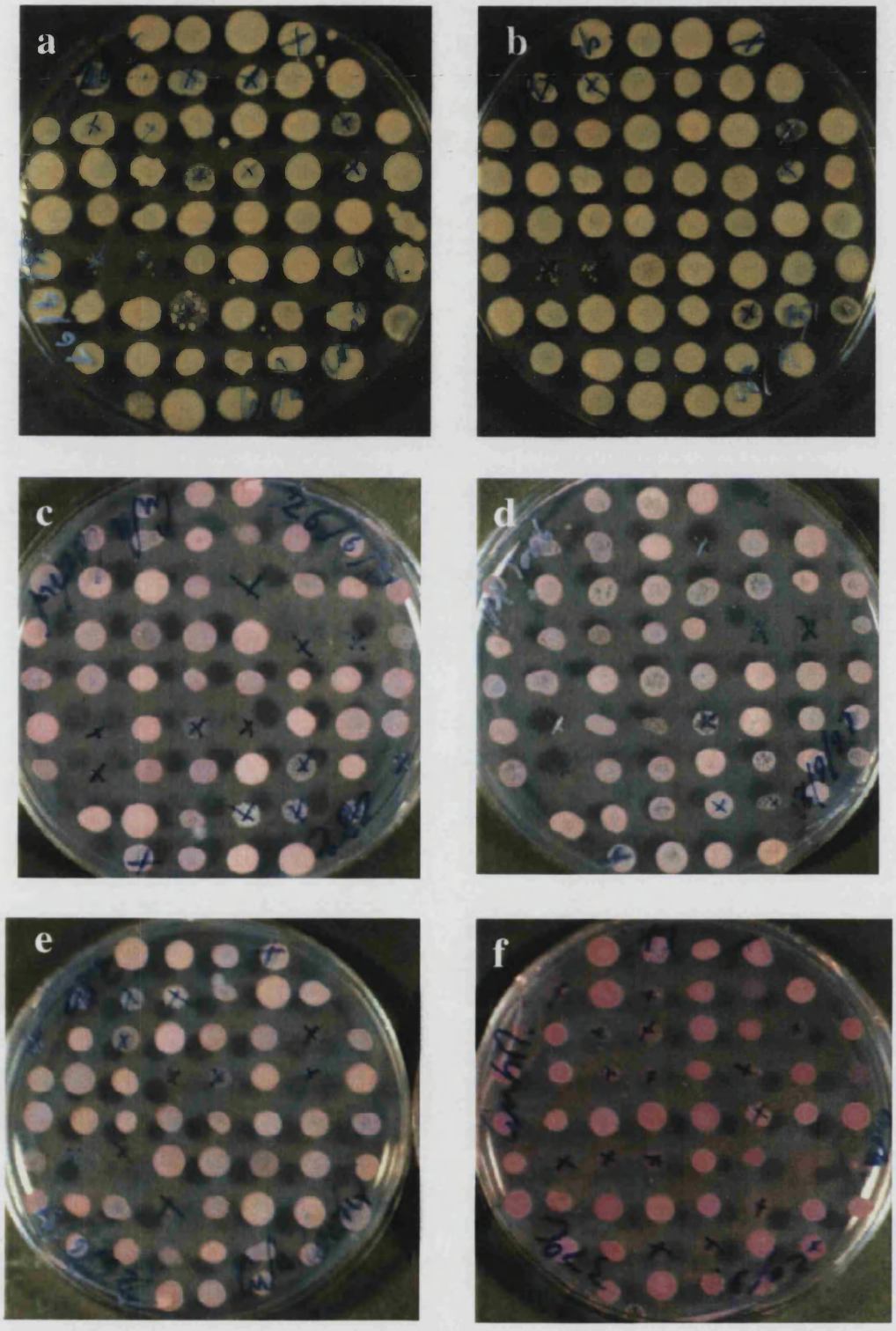
The objective of this research was to conduct a primary screen on a library of cell wall mutants with a similar genetic background in order to isolate strains with reduced viability in stationary phase for further assessment.

Results and discussion.

3.1 Cell wall carbohydrate composition in relation to viability in stationary phase.

Viability in stationary phase was assessed by growing strains in conditions where they would soon become deprived of glucose. *Cwh* strains were grown in liquid YPD containing 0.3% (w/v) glucose at 28°C with or without 1 M sorbitol in Falcon tubes on a shaker and then incubated at various temperatures (14°C, 28°C and 37°C). Cell viability was determined by drop tests on either solid YPD (2% (w/v) glucose) or SC (2% (w/v) glucose) media containing the vital stain, Phloxine B (Fig. 3.1.1). Phloxine B is a viability stain that is excluded by living cells but diffuses into dead cells. Therefore colonies that contain a high number of lysed or dead cells will be bright pink whereas colonies that contain mainly viable cells will be pale pink (Fig. 3.1.1). The main advantage of using Phloxine B in this study is that it can be used to screen a large number of mutants simultaneously under identical growth conditions. Additionally, it allows slow

Figure 3.1.1: Drop tests to isolate stationary phase phenotypes. Cells were grown with (**b, d**) or without 1M sorbitol (**a, c, e, f**) and then plated onto solid YPD (**a, b**) or solid SC media containing Phloxine B, 0.1 mg/ml (**c-f**). They were then incubated for 24-48 hrs at 14°C, 28°C (**e**) or 37°C (**f**).



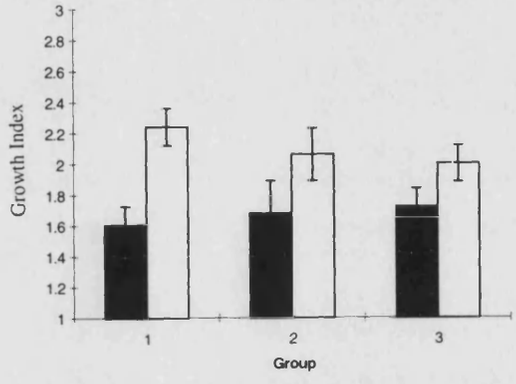
growing colonies to be distinguished from colonies that have low viability. The disadvantage of assessing growth in this manner is that results are qualitative rather than quantitative.

By dividing the mutants into three groups according to cell wall polysaccharide content it was possible to show how cell wall composition influenced the viability of cells during starvation (Fig.3.1.2). In general, cell wall composition was found to influence cell viability in stationary phase. Cells with low mannose content had reduced stationary phase viability when grown at 28°C in low glucose media. Cell viability significantly improved when they were grown with an osmotic stabiliser (Fig.3.1.2). At 28°C all groups had a higher growth index when grown with 1 M sorbitol added to the media. The influence of temperature and osmolarity on cell growth is further discussed in sections 3.2 and 3.4 respectively.

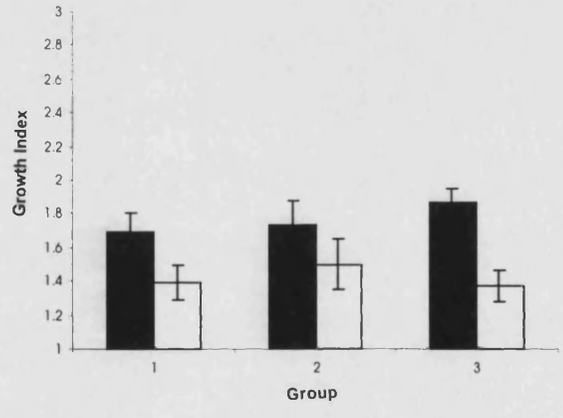
Overall, 47 % of strains displayed low growth or loss of viability after 3 weeks, this increased by a further 28 % over 5 weeks (Table 3.1.2 (a-f)). Recovery from starvation was influenced by incubation temperature and growth conditions. When cell viability was assessed by incubating at 28°C on solid YPD (2% (w/v) glucose) after 1 weeks growth in liquid YPD (0.3% (w/v) glucose, 28°C), several strains (16 strains) showed signs of reduced viability (Table 3.1.2 (a)). At this stage cells would either be in post-diauxic shift or entering stationary phase. Over half of the strains with reduced viability belong to group 1. After 5 weeks growth under the same conditions only a further six strains showed reduced

Figure 3.1.2: Viability assessment of cells grown to stationary phase in YPD (0.3% (w/v) glucose) liquid media incubated at 28 °C and then transferred onto SC (2% (w/v) glucose) media containing phloxine B and incubated at **a**) 28 °C, **b**) 37 °C and **c**) 14 °C. Cells incubated with (□) and without (■) 1M sorbitol. Group 1 (0.16-0.83 Man/Glc cell wall content) *n*=66, group 2 (0.84-1.18 Man/Glc cell wall content) *n*=34 and group 3 (1.20-3.92 Man/Glc cell wall content) *n*=54. Mean ± SEM of growth index 0 (no viability) to 3 (high viability) after 35 days growth .

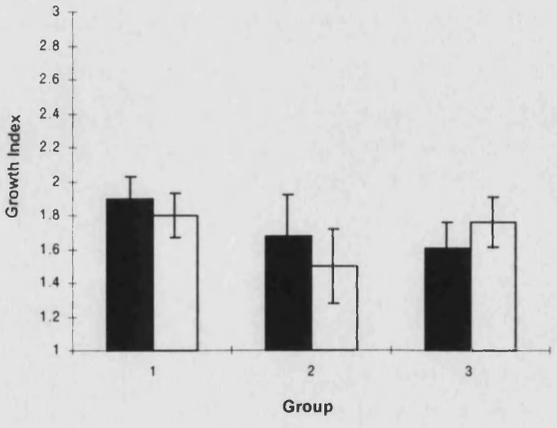
a)



b)



c)



viability. When strains were grown under the same conditions but incubated at 37°C a similar number (14 strains) lost viability after one weeks growth (Table 3.1.2 (b)). Again over half of these were from group 1. After 3 weeks only a further three strains lose viability but following 5 weeks growth this number increases by a further eight. A greater number of strains lose viability in long term stationary phase when they are incubated at 37°C then when they are incubated at 28°C. When strains are incubated at 14°C a different pattern emerges (Table 3.1.2 (c)). The same number of strains lose viability after 1 week as after 3 weeks (9 strains). In this case, group 1 strains do not have the greatest loss of viability as all groups are equally represented. In long term stationary phase a higher number of strains lose viability. After 5 weeks growth 11 strains lose viability, the majority of these are from group 1 (8 strains).

When strains are grown with 1 M sorbitol added to liquid YPD (0.3% (w/v) glucose, 28°C) and then tested for viability on solid YPD (2% (w/v) glucose) the growth pattern of strains is altered. In a similar way to those grown in low osmolarity, at 28°C the majority of strains show a reduction in growth after 1 week with fewer showing reduced growth after 3 and 5 weeks (Table 3.1.2 (d)). However, in contrast with strains that are grown without 1M sorbitol, after 1 weeks growth group 1 strains are not the least viable. Alternatively, there is an increase in the number of strains that lose viability from group 3. At 37°C incubation there is a greater number of strains after 1 week (16 strains) and 3 weeks (21 strains) that lose viability (Table 3.1.2 (e)). In this instance, over two thirds of these belong to group 1. Therefore, when cells are grown with 1 M sorbitol and then incubated at high temperatures, the protective properties that

Table 3.1.2 (a). Strains that lose viability in stationary phase after 7, 21 and 35 days growth in YPD (0.3% (w/v) glucose) and then incubated at 28°C on solid YPD (2% (w/v) glucose).

Strain	ORF/Gene†	Group	Phenotype††	Sporulation/Cell Division †††	Temp. sensitivity
Strains with reduced viability at 28°C following 7 days incubation					
cwh5		1	Zymolyase hs		
cwh7		1			
cwh9		1	Zymolyase hs		
cwh11		1	Zymolyase hs		
cwh13-1	*ERD1*	1	Zymolyase hs, Kre res		
cwh16		1			
cwh19		1	Caffeine hs	Spo⁻	
cwh23		1			
cwh25		1		Abnormal budding and cell lysis. Low sporulation	
cwh33		2			
cwh39		2			
cwh44-1		2	Zymolyase hs		
cwh47	*PTC1*	3		G1 arrest.	Ts lytic, cs
cwh48	*KRE6*	3	Killer res		
cwh49		3		Spores glusulase s. Germ⁻	
cwh51		3	Papulacandin B hs Caffeine hs		
Strains with reduced viability at 28°C following 21 days incubation.					
cwh2-1	*VRG1*	1	Zymolyase hs, Vandate Res, NaCl hs		
cwh17	*URE2*	2	Caffeine hs		
cwh22		2	Zymolyase hs Papulacandin B hs		
cwh34		3	Zymolyase hs, NaCl hs		
Strains with reduced viability at 28°C following 35 days incubation.					
cwh3		1	Zymolyase hs Papulacandin B hs		
cwh41	*GLS1*	3	Killer res	Budding defect	

† Previously characterised and cloned genes (*Saccharomyces* Genome Database).

†† Phenotypic tests involving Zymolyase, Papulacandin B, Caffeine, K1 killer toxin, temperature sensitivity and Rho from Ram *et al* (1994). NaCl sensitivity from Naik (1998).

††† Assessment of sporulation and cell division, unpublished results M Breitenbach (1997).

Table 3.1.2 (b). Strains that lose viability in stationary phase after 7, 21and 35 days growth in YPD (0.3% (w/v) glucose) and then incubated at 37°C on solid YPD (2% (w/v) glucose).

Strain	ORF/Gene†	Group	Phenotype††	Cell Division/ Sporulation†††	Temp sensitivity
Strains with reduced viability at 37°C following 7 days incubation.					
cwh1	*MNN9*	1	Zymolyase hs, NaCl hs		
cwh2-1	*VRG1*	1	Vandate res, Zymolyase hs, NaCl hs		
cwh3		1	PapulacandinB hs, Zymolyase hs		
cwh4	*GPI1*	1	Zymolyase hs, NaCl hs	Mating defective Spo$^-$, Germ$^-$	Ts lytic
cwh15		1			
cwh17	*URE2*	1	Caffeine hs		
cwh19		1	Caffeine hs	Spo$^-$	
cwh22		1	Caffeine hs, PapulacandinB		
cwh28		2			
cwh32	*VPS16*	2	Caffeine hs, PapulacandinB		
cwh36	*CWH36*	2	NaCl hs	Spo$^-$	
cwh37		2	Papulacandin B hs.		
cwh47	*PTC1*	3	Killer res.	G1 arrest.	Ts lytic
cwh53-1	*FKS1*	3	Papulacandin B hs, NaCl hs	Budding abnormality.	Ts lytic
Strains with reduced viability at 37°C following 21 days incubation.					
cwh42		3	Zymolyase hs, NaCl hs		
cwh50	*PLC1*	3			Ts lytic.
cwh51		3	Papulacandin B hs, Caffeine hs.		
Strains with reduced viability at 37°C following 35 days incubation.					
cwh6	*SPT14/GP13*	1	Zymolyase hs.		Ts lytic.
cwh9		1	Zymolyase hs.		
cwh12		1	Papulacandin B hs.		
cwh13-1	*ERD1*	1	Zymolyase hs, Killer res.		
cwh29		2	Zymolyase hs, Papulacandin B hs, Na Cl hs		
cwh41	*GLC1*	3	Killer res.		
cwh46-1		3	Zymolyase hs, Caffeine hs, Papulacandin B hs, Rho$^-$.	Mating defective	
cwh52-1	*GAS1*	3		Budding abnormality.	

† Previously characterised and cloned genes (*Saccharomyces* Genome Database).

†† Phenotypic tests involving Zymolyase, Papulacandin B, Caffeine, K1 killer toxin, temperature sensitivity and Rho from Ram *et al* (1994). NaCl sensitivity from Naik (1998).

††† Assessment of sporulation and cell division, unpublished results M Breitenbach (1997).

Table 3.1.2 (c). Strains that lose viability in stationary phase after 7, 21 and 35 days growth in YPD (0.3% glucose) and then incubated at 14°C on solid YPD (2% glucose).

Strain	ORF/Gene†	Group	Phenotype††	Cell Division/ Sporulation†††	Temp sensitivity
Strains with reduced viability at 14°C following 7 days incubation.					
cwh3		1	Zymolyase hs, Papulacandin B hs		
cwh4	*GPI1*	1	Zymolyase hs, NaCl hs	Mating defective Spo⁻, Germ⁻	Ts lytic.
cwh19		1	Caffeine hs.		
cwh36		2	NaCl hs	Spo⁻	
cwh39		2			
cwh47	*PTC1*	3	Killer res.		Ts lytic, cs.
cwh50	*PLC1*	3			Ts lytic.
cwh51		3	Papulacandin B hs, Caffeine hs.		
cwh53-1	*FKS1*	3	Papulacandin B hs, NaCl hs	Budding abnormality	Ts lytic
Strains with reduced viability at 14°C following 21days incubation.					
cwh15		1			
cwh17	*URE2*	1	Caffeine hs.		
cwh20		1	Zymolyase hs, Papulacandin B hs.		
cwh25		2		Abnormal budding and cell lysis, Low sporulation	
cwh32	*VPS16*	2	Caffeine hs, Papulacandin B hs.		
cwh37		2	Papulacandin hs.		
cwh40		2	Zymolyase hs.		
cwh44-1		3	Zymolyase hs.		
cwh46-1		3	Zymolyase hs, Caffeine hs, Papulacandin B hs, Rho⁻.	Mating defective	
Strains with reduced viability at 14°C following 35 days incubation.					
cwh1	*MNN9*	1	Zymolyase hs, NaCl hs		
cwh5		1	Zymolyase hs.		
cwh6	*SPT14/GPI3*	1	Zymolyase hs.		Ts lytic.
cwh8	*CAX4*	1	Zymolyase hs.		
cwh10		1	Zymolyase hs, Caffeine hs.		
cwh12		1	Papulacandin B hs.		
cwh13-1	*ERD1*	1	Zymolyase hs, Killer res.		
cwh21		1			
cwh22		2	Papulacandin B hs, Caffeine hs.		
cwh28		2			
cwh34		2	Zymolyase hs, NaCl hs		

† Previously characterised and cloned genes (*Saccharomyces* Genome Database).

†† Phenotypic tests involving Zymolyase, Papulacandin B, Caffeine, K1 killer toxin, temperature sensitivity and Rho from Ram *et al* (1994). NaCl sensitivity from Naik (1998).

††† Assessment of sporulation and cell division, unpublished results M Breitenbach (1997).

Table 3.1.2 (d) Strains that lose viability in stationary phase after 7, 21 and 35 days growth in YPD (0.3% (w/v) glucose) with 1M sorbitol then incubated at 28°C on solid YPD (2% (w/v) glucose).

Strain	ORF/Gene†	Group	Phenotype††	Budding/Cell Division†††	Temp sensitivity
Strains with reduced viability at 28°C following 7 days incubation in 1M sorbitol.					
cwh9		1	Zymolyase hs.		
cwh17	*URE2*	1	Caffeine hs.		
cwh19		1	Caffeine hs.	Spo$^-$	
cwh23		2			
cwh25		2		Abnormal budding and cell lysis. Low sporulation	
cwh33		2			
cwh39		2			
cwh41	*GLS1*	3	Killer res.		
cwh44-1		3	Zymolyase hs.		
cwh46-1		3	Zymolyase hs, Caffeine hs, Papulacandin B hs, Rho$^-$.	Mating defective	
cwh48	*KRE6*	3	Killer res		
cwh51		3	Caffeine hs, Papulacandin B.		
cwh53-1	*FKS1*	3	Papulacandin B, NaCl hs	Abnormal budding	
Strains with reduced viability at 28°C following 21 days incubation in 1M sorbitol.					
cwh27		2	Rho$^-$.		
cwh37		2	Papulacandin B hs.		
Strains with reduced viability at 28°C following 35 days incubation in 1M sorbitol					
cwh3		1	Zymolyase hs, Papulacandin B hs.		
cwh4	*GPI1*	1	Zymolyase hs, NaCl hs	Mating defective Spo$^-$, Germ$^-$	
cwh8	*CAX4*	1	Zymolyase hs.		
cwh36	*CWH36*	2	NaCl hs		

† Previously characterised and cloned genes (*Saccharomyces* Genome Database).

†† Phenotypic tests involving Zymolyase, Papulacandin B, Caffeine, K1 killer toxin, temperature sensitivity and Rho from Ram *et al* (1994). NaCl sensitivity from Naik (1998).

††† Assessment of sporulation and cell division, unpublished results M Breitenbach (1997).

Table 3.1.2 (e) Strains that lose viability in stationary phase after 7, 21 and 35 days growth in YPD (0.3% (w/v) glucose) with 1M sorbitol then incubated at 37 °C on solid YPD (2% (w/v) glucose).

Strain	ORF/Gene†	Group	Phenotype††	Cell Division/ Sporulation†††	Temp sensitivity
Strains with reduced viability at 37°C following 7 days incubation in 1M sorbitol.					
cwh1	*MMN9*	1	Zymolyase hs, NaCl hs		
cwh2-1	*VRG1*	1	Zymolyase hs, Vandate res, NaCl hs		
cwh4	*GPI1*	1	Zymolyase hs, NaCl hs	Mating defective Spo⁻, Germ⁻	Ts lytic
cwh11		1	Zymolyase hs.		
cwh12		1	Papulacandin B hs.		
cwh14		1	Caffeine hs.		
cwh15		1			
cwh16		1			
cwh18		1	Rho⁻, Papulacandin B hs.	Spore wall defective	
cwh19		1	Caffeine hs.	Spo⁻	
cwh20		1	Papulacandin B hs, Zymolyase hs.		
cwh25		2		Abnormal budding and cell lysis. Low sporulation	
cwh27		2	Rho⁻		
cwh37		2	Papulacandin B hs.		
cwh41	*GLS1*	3	Killer res		
cwh42		3	Zymolyase hs, NaCl		
Strains with reduced viability at 37°C following 21 days incubation in 1M sorbitol.					
cwh3		1	Papulacandin B hs, Zymolyase hs.		
cwh5		1	Zymolyase hs.		
cwh6	*SPT14/GPI3*	1	Zymolyase hs.		Ts lytic
cwh7		1			Ts lytic
cwh8	*CAX4*	1	Zymolyase hs.		
cwh9		1	Zymolyase hs.		
cwh10		1	Zymolyase hs, Caffeine hs.		
cwh21		1			
cwh22		2	Papulacandin B hs, Caffeine hs.		
cwh23		2			
cwh29		2	NaCl hs		
cwh32	*VPS16*	2	Papulacandin B hs, Caffeine hs.		
cwh34		2	Zymolyase hs, NaCl hs		
cwh36	*CWH36*	2	NaCl hs	Spo⁻	
cwh43-1		3			

(continued overleaf)

Table 3.1.2 (e) continued.

cwh44-1		3	Zymolyase hs		
cwh45		3		Spores glusulase s	
cwh46-1		3	Zymolyase hs, Caffeine hs, Papulacandin B hs, Rho⁻.		
cwh48	*KRE6*	3	Killer res.		
cwh52-1	*GAS1*	3		Abnormal budding, Germ⁻	
cwh53-1	*FKS1*	3	Papulacandin B hs, NaCl hs	Abnormal budding	Ts lytic
Strains with reduced viability at 37°C following 35 days incubation in 1M sorbitol.					
Cwh17	*URE2*	1			
cwh28		2			
cwh33		2			

† Previously characterised and cloned genes (*Saccharomyces* Genome Database).

†† Phenotypic tests involving Zymolyase, Papulacandin B, Caffeine, K1 killer toxin, temperature sensitivity and Rho from Ram *et al* (1994). NaCl sensitivity from Naik (1998).

††† Assessment of sporulation and cell division, unpublished results M Breitenbach (1997).

Table 3.1.2 (f). Strains that lose viability in stationary phase after 7, 21 and 35 days growth in YPD (0.3% (w/v) glucose) with 1M sorbitol and then incubated at 14 °C on solid YPD (2% (w/v) glucose).

Strain	ORF/Gene†	Group	Phenotype††	Cell Division/ Sporulation†††	Temp sensitivity
Strains with reduced viability at 14°C following 7 days incubation in 1M sorbitol					
cwh1	*MNN9*	1	Zymolyase hs, NaCl hs		
cwh4	*GPI1*	1	Zymolyase hs, NaCl hs	Mating defective Germ⁻, Spo⁻	Ts lytic
cwh18		1	Papulacandin B hs, Rho⁻		
cwh19		1	Caffeine hs.	Spo⁻	
cwh20		1	Zymolyase hs, Papulacandin B hs.		
cwh26	*VMA1*	2	Zymolyase hs, Caffeine hs, Papulacandin B hs.	Spo⁻	
cwh29		2	NaCl hs		
cwh36	*CWH36*	2		Spo⁻	
cwh46-1		3	Zymolyase hs, Caffeine hs, Papulacandin B hs, Rho⁻.	Mating defective	
Strains with reduced viability at 14°C following 21 days incubation in 1M sorbitol.					
cwh3		1	Zymolyase hs, Papulacandin B hs.		
cwh5		1	Zymolyase hs.		
cwh6	*SPT14/GPI3*	1	Zymolyase hs.		Ts lytic.
cwh7		1			Ts lytic.
cwh12		1	Papulacandin B hs.		
cwh14		1	Caffeine hs.		
cwh16		1			
cwh17	*URE2*	1	Caffeine hs.		
cwh22		2	Papulacandin B hs, Caffeine hs.		
cwh23		2			
cwh25		2		Abnormal budding and cell lysis. Low sporulation	
cwh27		2	Rho⁻.		
cwh34		2	Zymolyase hs, Na Cl hs		
cwh43-1		3			
cwh52-1	*GAS1*	3		Abnormal budding Germ⁻	
cwh53-1	*FKS1*	3	Papulacandin B hs, NaCl hs	Abnormal budding	Ts lytic.
Strains with reduced viability at 14°C following 35 days incubation in 1M sorbitol.					
cwh2	*VRG1*	1	Zymolyase hs, Vandate res, NaCl hs		
cwh13-1	*ERD1*	1	Zymolyase hs, Killer res.		
cwh28		2			
cwh33		2			
cwh44-1		3	Zymolyase hs.		
cwh47	*PTC1*	3	Killer res.		Ts lytic.
cwh49		3		Spores glusulase s Germ⁻	
cwh50	*PLC1*	3			Ts lytic.

† Previously characterised and cloned genes (*Saccharomyces* Genome Database).

†† Phenotypic tests involving Zymolyase, Papulacandin B, Caffeine, K1 killer toxin, temperature sensitivity and Rho from Ram *et al* (1994). NaCl sensitivity from Naik (1998).

††† Assessment of sporulation and cell division, unpublished results M Breitenbach (1997).

high osmolarity offers group 1 at optimum growth temperatures are cancelled out. In cells that have been growing for three weeks then incubated at 37°C the greatest number of strains lose viability but these are represented equally by all three groups. When strains are incubated at 14°C, as in those incubated at 37°C there is the greatest reduction in viability after 3 weeks (17 strains) (Table 3.1.2 (f)). A fewer number of strains lose viability after 1 week (7 strains) and 5 weeks (8 strains).

These results seem to illustrate the protective properties of mannoproteins and emphasise the way they enable cells to survive extreme environments. When cells are grown in low glucose and low osmolarity and then incubated at 28°C, the cells with the least ability to grow are those from group 1, strains with a low mannose content. After 7 days growth they either lose viability in liquid media or are not able to adapt to growth on solid YPD (2% (w/v) glucose). Grown in the presence of 1M sorbitol, the viability of group 1 cells improves when transferred to solid YPD (2% (w/v) glucose) at 28°C. This is not the case when cells are re-incubated at 37°C. Therefore, when group 1 cells are grown in 1M sorbitol their physiological state may change in a way that improves growth at 28°C but prevents them from adapting to higher temperatures. Cells grown in high osmolarity may have increased intracellular glycerol and trehalose content. They may also have an increased level of cell wall proteins (CWPs). If trehalose and protein levels are higher then an increased resistance to stress would be expected. Group 1 cells would therefore not show reduced viability when incubated at elevated temperatures. In further work carried out in this study (Chapter 4) it was discovered that trehalose levels are often associated with zymolyase resistance.

Strains that are sensitive to Zymolyase often have reduced intracellular trehalose content (Chapter 4, 4.2.2). Group 1 contains the highest number of Zymolyase-sensitive cells therefore it probably also has a high number of strains with reduced intracellular trehalose levels. An alternative explanation is probably that 1M sorbitol may just osmotically correct the lysis effect caused by a weakened cell wall without greatly altering the physiological state of the cell. A number of group 1 mutants are known to have mannosylation and secretory defects (e.g., *cwh1/mnn9, cwh13/erd1*). This could inhibit the synthesis of proteins needed to protect the cell from elevated temperatures. Cells with a wild type ratio of mannose and glucose polymers appeared to adjust to the external environment the most effectively giving the least variation in growth, under the conditions assessed (Fig.3.1.2).

3.2 Influence of temperature on cell cycle re-entry.

As previously discussed (section 3.1) each group gave different patterns of recovery from stationary phase when incubated at different temperatures. Strains were grown up to five weeks in liquid YPD (0.3% (w/v) glucose, 28°C), transferred to solid YPD (2% (w/v) glucose) and re-incubated at a specific temperature (14°C, 28°C and 37°C). The greatest reduction in viability was at the highest temperature of 37°C (Fig. 3.2.1).

High temperatures are known to exacerbate defects in cell wall construction causing cell lysis (Zhao *et al.*, 1998). This effect was most prominent in group 1 strains with the lowest mannose content (Fig.3.2.1). Cell wall proteins and storage carbohydrates, such as trehalose, play an important role in adaptation to growth at high temperatures (Winkler *et al.*, 1991). Cell wall proteins give cells

additional protection from stress. Group 3 strains with the highest mannose content were least affected by an increase in temperature.

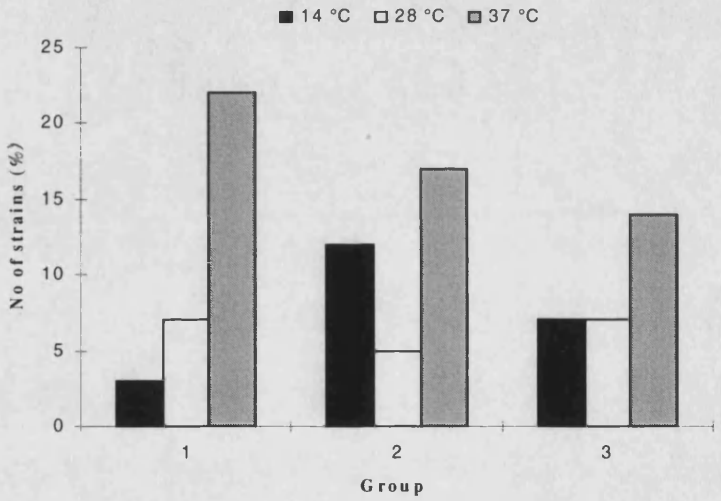
A number of the *cwh* strains are already known to have a temperature sensitive phenotype (Ram *et al.*, 1994). At 37°C and 14°C a number of strains that have a temperature sensitive phenotype lose viability (Table 3.1.2 (a-f)). At 28°C there is only one temperature sensitive strain that loses viability when grown without 1M sorbitol and no temperature sensitive strains lose viability at 28°C when grown with 1M sorbitol. At cold temperatures (14°C) cells with a wild phenotype (Group 2) were the only group with reduced viability. Overall there was no significant difference in viability between the three groups. Cold sensitivity is most often associated with defects in assembly of multi-subunit complexes because these processes are often driven by entropy (Hampsey, 1997). As the polysaccharide content in group 2 is similar to wild type cells, cell cycle defects or the arrangement of polysaccharides in the cell wall may be a contributing factor to calcofluor-white sensitivity. Examples of genes amongst group 2 of the *cwh* strains with known function are *VMA1* and *VPS16*. Both of these genes are involved in vacuolar processes. *VMA1* encodes a subunit for a vacuolar trifluoperazine resistance protein that aggregates with other molecules and is cold sensitive (Kawasaki *et al.*, 1997). The null mutant is defective in starvation-induced degradation of proteins and accumulates autophagic vesicles in the vacuole under starvation conditions (Nakamura *et al.*, 1997). *VPS16* encodes a protein that is involved in vacuolar protein sorting. The null mutant is temperature sensitive for growth and displays abnormal vacuolar and cell morphology (Wada *et al.*, 1992).

The temperature sensitive strains that show up most frequently in the screen are *cwh4/gpi1, cwh6/gpi3, cwh7, cwh47/ptc1, cwh50/plc1* and *cwh53-1/fks1*. Most of the affected genes are directly involved in cell wall construction. *CWH4/GPI1* and *CWH6/GPI3* are involved in the construction of GPI anchors. *GPI1* participates in the synthesis of the first intermediate in the synthesis of glycosylphosphatidylinositol (GPI) anchors (Leidich and Orlean, 1996). This particular strain lost viability in all growth conditions when incubated at a temperature that deviated from 28°C. It forms multibudded cells at these temperatures in a similar way to *cwh47/ptc1*. *CWH6/GPI3* is both osmotically and temperature sensitive. *GPI3* mutants are defective in synthesis of GlcNAc-phosphatidylinositol, which is involved in the first step of GPI anchor synthesis (Schonbachler *et al.*, 1995). Both *GPI1* and *GPI3* are essential for growth, as deletions in these genes are lethal (MIPS database, 2000).

There is no published information available on *cwh7* but the remaining temperature sensitive strains, *cwh47/ptc1*, *cwh50/plc1* and *cwh53/fks1* have been well studied (Jiang *et al.*, 1995; Flick and Thorner, 1993; Ram *et al.*, 1995). *PTC1* encodes a protein serine-threonine phosphatase that is involved in osmoregulation. Although this strain is temperature sensitive without 1M sorbitol this defect is corrected when 1M sorbitol is added to the media. However, this strain also lost viability at 28°C therefore the osmolarity of the growth media is probably the predominant factor in cell viability. The *PLC1* gene product is a phosphoinositide-specific phospholipase C that could be involved in the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP2) to generate two second messengers, inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG). The hydrolysis of PIP2 in *S. cerevisiae* is required for a number of nutritional and

stress-related responses (Flick and Thorner, 1993). The *PLC1* gene causes a chromosome segregation defect that is partially suppressed by adding calcium to the growth media (Flick and Thorner, 1993). This study has found that *PLC1* is temperature sensitive only in hyperosmotic conditions. These findings replicate published results (Payne and Fitzgerald-Hayes, 1993). *FKS1* encodes a gene which functions as a transferase that is involved in carbohydrate metabolism and β1,3-glucan synthesis (Douglas *et al.*, 1993; Ram *et al.*, 1995).

Figure 3.2.1: Total number of strains that showed reduced viability after 5 weeks growth at 28°C in liquid YPD media (0.3% (w/v) glucose) then reincubated at specified temperatures on solid media for 24-48 hours to assess cell viability in stationary phase.



Overall, it seems that cell separation is a contributing factor to temperature sensitivity in all of these strains. *CWH4/GPI1, CWH6/GPI3, CWH47/PTC1, CWH50/PLC1* and *CWH53/FKS1* all have phenotypic defects that affect cell division and separation. Therefore, the ability of the cell to adapt to a stationary phase morphology is a contributing factor to survival in extreme conditions.

3.3 Influence of phenotype on cell viability in stationary phase.

The *cwh* strains had already been characterised by several biophysical assays and attributed with a variety of different phenotypes (Ram *et al.*, 1994) (Table 3.0.1). These phenotypes exert different influences on viability depending on growth conditions. For example, Zymolyase hypersensitivity reduces viability during stationary phase in low glucose and osmolarity medium but this phenotype is suppressed in many cases when 1M sorbitol is added as an osmotic stabiliser (Fig.3.4.1). Phenotypes that had previously been determined by Ram *et al.* (1984) were assessed for loss of viability under the conditions used to isolate stationary phase mutants in this study (Table 3.3.1).

3.3.1 Zymolyase sensitivity.

Originally, Ram *et al.* (1994) isolated 19 strains with Zymolyase-hypersensitive phenotypes from a library of *cwh* strains (Table 3.0.1). Most of these were found in the low mannose group (Group 1). Seven of these were also in a group of 20 isolated in the screen for reduced viability in long term stationary phase (*cwh3, cwh5, cwh10, cwh13-1, cwh26, cwh42* and *cwh46-2*).

When Zymolyase-sensitive strains were grown in liquid YPD (0.3% (w/v) glucose, 28°C) then incubated on solid YPD (2% (w/v) glucose) at 28°C, the greatest reduction in viability was seen after 1 week (*cwh5, cwh9, cwh11, cwh13, cwh44*). Most of these strains were in group 1 (Table 3.1.2 (a)). After 5 weeks only a further four strains lost viability (*cwh2, cwh3, cwh22, cwh34*). When Zymolyase-sensitive strains were grown and incubated under similar conditions with 1M sorbitol added to the liquid media only three strains lost viability after 1 week (*cwh9, cwh44, cwh46*) (Table 3.1.2 (d)). Only one of these strains was in

Table 3.3.1: Number of *cwh* strains with additional phenotypes that show reduced viability in stationary phase. Cells were gown in liquid YPD (0.3% (w/v) glucose) with or without 1M sorbitol and then incubated at 28°C, 37°C and 14°C on solid YPD (2% (w/v) glucose). The *cwh* strains have been previously screened for other phenotypes by Ram *et al.* (1994) and Naik, (1998).

***Phenotype*†:**	**Zymolyase sensitive**	**NaCl sensitive**	**Papulacandin B sensitive**	**Caffeine sensitive**	**Rho⁻**	***K1* Killer resistant**
Total No of phenotypes in cwh strains (n=53).	19	8	11	9	3	4
Growth conditions:						
YPD (0.3% (w/v) glucose) then incubated at 28°C.	8	2	3	3	0	3
YPD (0.3% (w/v) glucose) then incubated at 37°C.	9	7	9	6	1	3
YPD (0.3% (w/v) glucose) then incubated at 14 °C.	14	5	9	7	1	2
YPD (0.3% (w/v) glucose) + 1M sorbitol then incubated at 28°C.	6	3	5	4	2	1
YPD (0.3% (w/v) glucose) + 1M sorbitol then incubated at 37°C.	17	8	8	6	3	2
YPD (0.3% (w/v) glucose) + 1M sorbitol then incubated at 14°C.	13	7	8	6	3	2

† Phenotypic tests involving Zymolyase, Papulacandin B, Caffeine, K1 killer toxin, temperature sensitivity and Rho from Ram *et al.* (1994). NaCl sensitivity from Naik (1998).

group 1. Therefore 1M sorbitol seemed to correct this phenotype in all except two strains (*cwh9, cwh44*). This situation was reversed when Zymolyase-sensitive strains were incubated at 37°C. After growing for 1 week with 1M sorbitol added to the media then incubated at 37°C (Table 3.1.2(e)), six strains showed loss of viability (*cwh1, cwh2, cwh4, cwh11, cwh20, cwh42*). After 5 weeks growth a

further nine lost viability (*cwh3, cwh5, cwh6, cwh8, cwh9, cwh10, cwh34, cwh44, cwh46*). When grown in liquid media without 1M sorbitol and then incubated at 37°C only 4 strains lost viability (*cwh1, cwh2, cwh3, cwh4*) (Table 3.1.2 (b)). A further six strains lost viability after 5 weeks growth (*cwh6, cwh9, cwh13, cwh29, cwh46*). Therefore at 37°C, Zymolyase-sensitive cells that have been grown with 1M sorbitol added to the media have reduced tolerance to high temperatures,

When Zymolyase-sensitive strains were incubated at 14°C following 1 week's growth in liquid YPD (0.3% (w/v) glucose, 28°C) only 2 strains showed a reduction after 1 week (*cwh3, cwh4*). This increased to five when 1M sorbitol was added to the media (*cwh1, cwh4, cwh20, cwh26, cwh46*). Without 1M sorbitol after five weeks a further 11 strains showed loss of viability (*cwh1, cwh5, cwh6, cwh8, cwh10, cwh13, cwh20, cwh34, cwh40, cwh44, cwh46*). When grown with 1M sorbitol only a further 7 strains showed reduced viability (*cwh2, cwh3, cwh5, cwh6, cwh13, cwh34, cwh44*). Overall the same number of strains lose viability within 5 weeks. Therefore increasing osmolarity at 14°C does not seem to have any effect on cell viabiliity.

Zymolyase is a β1,3-glucanase enzyme that can be used to detect increased permeability in the cell wall. Mutations which affect mannosylation of cell wall mannoproteins usually have a higher sensitivity to Zymolyase. Most of the zymolyase hypersensitive stationary phase mutants were in the low mannose group. Zymolyase sensitivity seems to be a contributing factor to loss of viability in stationary phase for *cwh* mutants in an environment which has a high

temperature and osmolarity and also with strains that are grown at low temperatures in high or low osmolarity (Table 3.3.1). At optimum temperatures high osmolarity appears to have a remedial effect on Zymolyase-sensitive strains. Cell lysis probably occurs in Zymolyase- hypersensitive strains at high temperatures combined with high osmolarity media because of additional factors imposed on the cell wall.

3.3.2 Salt sensitivity.

The *cwh* strains were also assessed for tolerance to salt (Naik, 1998). Salt (0.6M NaCl) stress prevented growth in eight strains at 28°C (Table 3.3.1). Three of these belonged to group 1 (*cwh1, cwh2, cwh4*), two were from group 2 (*cwh29, cwh34*) and the remaining two were in group 3 (*cwh42, cwh53*). Six of these were also sensitive to Zymolyase (Table 3.0.1).

NaCl-sensitive strains were assessed for viability in the stationary phase screen. They were first grown in liquid YPD (0.3% (w/v) glucose, 28°C) with or without 1M sorbitol then transferred to solid YPD (2% (w/v) glucose) and incubated at different temperatures (Table 3.1.2 (a-f)). When incubated at 28°C, only two of the salt sensitive strains showed reduced viability (*cwh2* and *cwh34*). In these two strains the defect was corrected with 1M sorbitol. At 28°C when grown in high osmolarity medium three different strains showed reduced viability (*cwh4, cwh36* and *cwh53*). A different pattern emerges when these strains are incubated at temperatures that deviate from the optimum. At 37°C five NaCl-sensitive strains show a reduction in viability after only one week's growth (*cwh1, cwh2, cwh4, cwh36, cwh53*). Seven, in total, showed reduced viability after 5 weeks. A similar pattern emerges when strains are grown in 1M sorbitol and then

incubated at 37°C. All of the eight NaCl-sensitive strains lose viability after 5 weeks. Half of these lose viability after 1 week's growth (*cwh1*, *cwh2*, *cwh4*, *cwh42*). The remaining four salt sensitive strains lose viability after 3 weeks (*cwh29*, *cwh34*, *cwh36*, *cwh53*). At 14°C incubation there is also an increased loss of viability in salt sensitive strains. When cells are grown in low glucose media with low osmolarity three strains lose viability after 1 week when incubated at 14°C (*cwh4*, *cwh36* and *cwh53*). The same number of strains lose viability at this temperature after 1 week when 1M sorbitol is added to the media (*cwh1*, *cwh4* and *cwh53*). In total at 14°C, five strains lose viability without an osmotic stabiliser added to the media and six strains lose viability with an osmotic stabiliser added to the media.

These results demonstrate that not only temperature but osmolarity influences the viability of salt sensitive strains. *cwh4* for instance shows increased sensitivity to changes in temperature. It also has slightly reduced viability in long term stationary phase at 28°C when 1M sorbitol is added to the media. The temperature sensitivity of *cwh4* was also isolated in the observations made by Ram *et al.* (1994). Similar results were found in *cwh36* and *cwh53*. These three temperature sensitive strains, *cwh4*, *cwh36* and *cwh53*, also showed reduced viability at 28°C when 1M sorbitol was added to the media. All three strains are in different groups. The expression of several genes is increased by high concentrations of NaCl, KCl or sorbitol. Amongst these is *HAL1* which is known to increase expression when grown in high osmolarity. In cells overexpressing *HAL1*, sodium toxicity is counteracted by an increased accumulation of potassium (Gaxiola *et al.*, 1992). The ability of yeast to maintain the balance of

transmembrane protons gradients and hydrogen ions involves H^+ translocating ATPase enzymes. A major constituent of the yeast cell membrane is H^+-ATPase. It controls intracellular pH, nutrient and ion transport and cell growth. Cells with a mutation in the ATPase gene have reduced growth rates, altered budding patterns and elongated cell morphology. Cells with defects that prevent the incorporation of this enzyme into the membrane may have a similar phenotype. The activity of this enzyme is reduced in stationary phase cells (Walker, 1999). Therefore stationary phase physiology probably dispenses with the necessity to regulate ion uptake. Salt sensitivity and reduced viability could occur in these cells because certain cell wall defects reduce the ability to regulate ion transportation.

Amongst the group 3 mutants that were hypersensitive to salt was *cwh53-1* which is allelic to the *FKS1* gene. *FKS1* mutants are sensitive to FK506 (Eng *et al.*, 1994). FK506 is an immunosuppressive agent that acts by blocking a Ca^{2+}-dependent signal transduction pathway leading to interleukin-2 transcription. The drug forms a complex with Ca^{2+}/calmodulin-dependent protein phosphatase 2B (PP2B), or calcineurin. Although PP2B is not essential for growth under normal conditions inhibition of growth occurs under certain stress conditions. PP2B is required for NaCl and LiCl resistance, but not for KCl, $CaCl_2$, $MgCl_2$ or non-specific osmotic stress (Nakamura *et al.*, 1993). Mutations in *FKS1* therefore, seem to disrupt the regulation of intracellular ions in yeast and adaptation to high salt stress conditions. When wild type cells are grown in the presence of FK506, growth behaviour and intracellular Na^+ and K^+ levels in high NaCl medium become similar to PP2B-deficient mutants in a manner dependent on the level of the FK506-binding Ca^{2+}-dependent protein (Nakamura *et al.*, 1993). Mutations

that produce a FK506 hypersensitive phenotype seem to have a similar effect to those that cause a reduction in PP2B activity.

Sensitivity to salt also seems to be exacerbated by variation in temperatures. The responses to certain stress elements could involve the same cell wall components. Many of the salt sensitive strains are also Zymolyase sensitive as previously discussed (section 3.3.1). Zymolyase sensitive strains are also more susceptible to high temperatures.

3.3.3 Papulacandin B sensitivity.

Ram *et al.* (1994) isolated eleven mutants with increased Papulacandin B sensitivity (Table 3.0.1). Five were in group 1, and three were in each of group 2 and 3. Of the *cwh* strains screened, temperature exerted the largest influence on the viability of Papulacandin B sensitive strains (Table 3.3.1).

Papulacandin B-sensitive strains showed the least reduction in viability when viability was assessed at optimal temperature (28°C) on solid YPD (2% (w/v) glucose). Just one strain (*cwh51*) showed a reduction of viability after 1 weeks growth in liquid YPD (0.2% (w/v) glucose). Only a further two strains (*cwh3, cwh22*) showed reduced viability after 5 weeks. A similar situation occurs when 1M sorbitol is added to the media. Three strains (*cwh46, cwh51, cwh53*) showed reduced viability after 1 week and a further two (*cwh3, cwh37*) after 5 weeks. When Papulacandin B-sensitive strains are subjected to a variation in temperature there is an increase in the number that lose viability. Following incubation at 37°C the number increases to five strains after 1 week (*cwh3, cwh22, cwh32, cwh37, cwh53*) and a further four strains after 5 weeks (*cwh51,*

cwh12, cwh29, cwh46). With 1M sorbitol added to the growth media this figure is four after 1 week (*cwh12, cwh18, cwh20, cwh37*) and five strains following 5 week's growth (*cwh3, cwh22, cwh32, cwh46, cwh53*). At an incubation temperature of 14°C this number is three strains after 1 week (*cwh3, cwh51, cwh53*) and a further 6 following 5 weeks growth (*cwh12, cwh20, cwh22, cwh32, cwh37, cwh46*). With 1M sorbitol added to the media this figure is 3 after 1 week (*cwh18, cwh20, cwh46*) and a further 4 after 5 weeks (*cwh3, cwh12, cwh22, cwh53*).

Papulacandin B is an anti fungal agent that interferes with the synthesis of β1,3-glucan (Kopecka, 1984; Castro *et al.*, 1995). In protoplast regeneration studies using electron microscopy, Papulacandin B was found to inhibit the biogenesis of β1,3-glucan fibrillar nets; leading to non-polar spherical growth (Kopecka, 1984). It inhibits specifically the synthesis of alkali-insoluble branched β1,3-glucan, a necessary building unit required for the formation of the fibrillar component of the cell wall responsible for cell shape, rigidity and tensile strength. These results indicate that this component involved in Papulacandin B resistance also plays a role in regulating the cell wall during variations in temperature

3.3.4 Caffeine sensitivity.

In total nine of the *cwh* strains were found to be sensitive to caffeine (Ram *et al.*, 1994) (Table 3.0.1). All of these were assessed for loss of viability in stationary phase (Table 3.3.1). Following 1 weeks growth in liquid YPD (0.3% (w/v) glucose, 28°C) only two strains (*cwh19, cwh51*) lost viability when transferred to solid YPD (2% (w/v) glucose) and incubated at 28°C (Table 3.1.2(a)). After 5

weeks under the same growth conditions just one more strain (*cwh17*) showed reduced viability. Under the same growth conditions but incubated at 37°C six caffeine sensitive strains showed reduced viability (Table 3.1.2. (b)). Of these four (*cwh17, cwh19, cwh22, cwh32*) showed reduced viability after 1 week's growth and a further two strains (*cwh51, cwh46*) lost viability following 5 week's growth. When incubated at 14°C under the same growth conditions seven strains showed reduced viability (Table 3.1.2.(c)). Two of these (*cwh19, cwh51*) lost viability after 1 week and a further five strains (*cwh10, cwh17, cwh22, cwh32, cwh46*) lost viability after 5 weeks.

When grown under the same conditions as above but with 1M sorbitol added to the media a total of four strains lost viability after1 week when incubated at 28°C (*cwh17, cwh19, cwh46, cwh51*). When strains were incubated at 37°C six strains lost viability over 5 weeks. Two of these (*cwh14, cwh19*) lost viability after 7 days and four strains (*cwh10, cwh22, cwh32, cwh46*) lost viability after 3 weeks. Following incubation at 14°C six strains lost viability overall. Three strains (*cwh19, cwh26, cwh46*) after 7 day's growth with 1M sorbitol and a further 3 strains (*cwh14, cwh17, cwh22*) after 3 week's growth.

One of the caffeine-sensitive strains that lost viability at all of the temperatures was a generally slow growing strain (*cwh19*). Two of the strains (*cwh22, cwh32*) only lost viability when the temperature deviated from optimum growth of 28°C. One strain (*cwh46*) lost viability due to changes in temperature and osmolarity.

Caffeine is a purine analogue that inhibits several cellular processes. It interferes with reactions involving cAMP phosphodiesterases causing background levels of cAMP to rise which in turn activates cAMP-dependent protein kinases. Caffeine sensitivity is most often associated with signal transduction mutations. Strains with mutations that affect the *PKC1/MPK1* signal transduction pathway often have increased sensitivity to caffeine. Of the 53 complementation groups tested nine were found to have increased sensitivity to caffeine (Ram *et al.*, 1994).

In the stationary phase screen high temperatures seemed to have the greatest influence on viability in caffeine-hypersensitive strains (Table 3.3.1). Although a combination of high osmolarity and low temperature also reduced viability in this phenotype. Yeast cells respond to a variety of stresses through a global stress response that is mediated by a number of signal transduction pathways and the cis-acting *STRE* DNA sequence. This global response is activated when cells are confronted with heat shock, glucose starvation, approach-to-stationary phase and osmotic stress (Evangelista *et al.*, 1996). Loss of viability in caffeine sensitive yeast cells subjected to extreme physical conditions would therefore be as a consequence of impaired signal transduction pathways in this particular phenotype.

3.3.5 K1 killer resistance.

Five strains (*cwh13, cwh30, cwh41, cwh47, cwh48*) from the *cwh* library were isolated by Ram *et al.* (1994) as *K1* killer resistant (Table 3.0.1). There were only four killer resistant phenotypes in all the *cwh* strains investigated as *cwh30* was

unavailable at the time of screening. After 7 day's growth (liquid YPD 0.3% (w/v) glucose, 28°C) followed by incubation at 28°C three of the killer resistant strains lose viability (*cwh13, cwh47, cwh48*). After 5 week's growth, the remaining strain (*cwh41*) shows reduced viability. Three of the killer resistant strains have reduced viability at 37°C. One strain (*cwh47*) after 7 day's growth and two strains (*cwh13, cwh41*) after 5 week's growth. Two strains have reduced growth at 14°C incubation (*cwh47, cwh13*).

When 1M sorbitol is added to the media loss of viability is corrected in some cases. At 28°C incubation only one strain lost viability (*cwh48*). At 37°C two strains lose viability (*cwh41, cwh48*). While at 14°C a different two strains lose viability (*cwh13, cwh47*). These results reveal that two strains (*cwh13, cwh47*) are sensitive to low osmolarity, a defect that is corrected by1M sorbitol.

The receptor site for the *K*1 killer toxin also binds to β1,6-glucan. Killer toxin resistance therefore indicates a defect in β1,6-glucan. All the killer resistant strains (*cwh13, cwh41, cwh47* and *cwh48)* have been complemented with killer resistant mutants (*kre1, kre2, kre5, kre6, kre9, kre10* and *kre11*) (Roemer and Bussey, 1991). *CWH48* was found to be allelic to *KRE6* which encodes a putative β1,6-glucan synthase component. The other killer resistant strains were complemented by all the *kre* mutants which indicated that they were not allelic but new killer resistant mutants (Ram, 1996).

Three of the killer resistant mutants, *cwh13, cwh41* and *cwh47*, become heat sensitive by week 5. In *cwh13* and *cwh47* this defect is corrected by the addition

of an osmotic stabiliser. In *cwh41* the addition of an osmotic stabiliser exacerbates the defect. All three of these mutants have been previously studied. *CWH13* is also known as *ERD1* which encodes a protein required for retaining luminal ER proteins (Hardwick *et al.*, 1990). *cwh13/erd1* has a low mannose wall content and is hypersensitive to zymolyase indicating increased cell wall permeability. Since mannoproteins are synthesised within the secretory pathway, a defect in this process could have a synergistic effect and leave the mannoproteins unable to bind with the cell wall matrix. The increased permeability would allow some glycoproteins that normally bind with β1,6-glucan, to be secreted into the surrounding media. As this condition is corrected by the addition of an osmotic stabiliser it indicates that the weak structure of the cell wall is an important factor in reducing viability. Additionally there could be alterations in the cell wall imposed by a high osmolarity that suppresses this phenotype.

3.3.6 Rho⁻

In all of the *cwh* strains only three were found to be Rho⁻, *cwh18, cwh27* and *cwh46-1* (Ram *et al.*, 1994) (Table 3.0.1). All of these were assessed for loss of viability in stationary phase. They where all grown in liquid YPD (0.3% (w/v) glucose, 28°C) then transferred to solid YPD (2% (w/v) glucose) and incubated at 28°C, 14°C and 37°C. Only one of these strains showed loss of viability under these screens at low osmolarity (*cwh46*). When strains were grown with 1M sorbitol added to the media a different pattern emerged with all of the Rho⁻ strains showing loss of viability. Following 3 week's growth and incubating at

37°C or 14°C all three strains had reduced viability. Two strains (*cwh46, cwh27*) lost viability after incubating at 28°C when they were grown with 1M sorbitol.

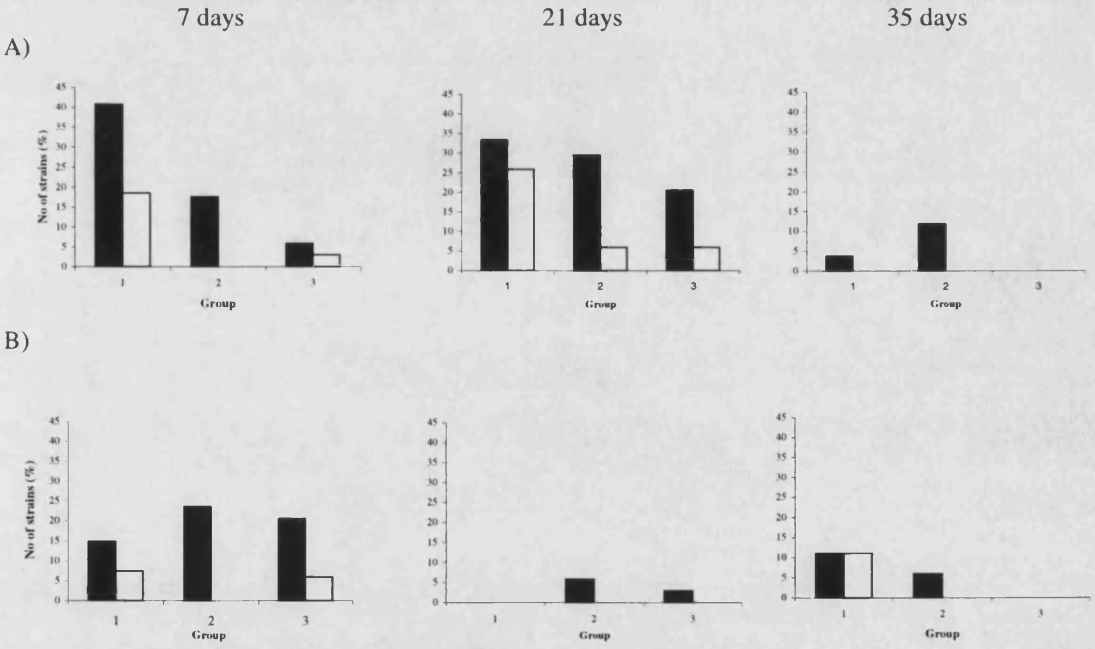
The *Rho* genes encode membrane associated Ras related GTP-binding proteins that are involved in the control of morphogenetic processes. Defects in the *Rho* genes result in cell cycle arrest at the small bud stage with concomitant cell lysis (Drgonová *et al.*, 1996). Loss of viability in *Rho*$^-$ strains appears to be most influenced by increased osmolarity (Table 3.3.1). *Rho* proteins are associated with osmotic regulation. Recent evidence indicates that Rho family small GTP-binding proteins regulate reorganization of the actin cytoskeleton (Tanaka and Takai, 1998). Actin reorganisation is an important part of the adaptation process yeast cells undertake in osmoregulation. Therefore, cells with a mutation that interferes with this process or any of the other steps in osmoregulation would probably lose viability in stationary phase. The cell wall defects resulting from this type of mutation can be aggravated by starvation (Verna *et al.*, 1997).

3.4 Influence of osmolarity on cell viability in *cwh* strains.

When yeast cells are grown in high osmolarity media, such as one with a high salt concentration, they adapt by accumulating glycerol to balance internal osmolarity. This mechanism is controlled by a Mitogen Activating Protein (MAP) cascade called the high osmotic glycerol (HOG) response pathway (Schuller *et al.*, 1994; Ram *et al.*, 1994). In response to low osmolarity a different MAP kinase pathway is activated called the *SLT2/MPK1* pathway (Davenport *et al.*, 1995).

A temporary cessation of growth and a transition effect is observed when cells are transferred into media with a different osmolarity. When cells are transferred from one environment to another, growth arrest is accompanied by a redistribution of actin filaments (Brewster and Gustin, 1994). Following a transition period actin filaments return to their previous distribution and cell division is resumed (Brewster and Gustin, 1994).

Fig 3.4.1: Number of strains with reduced growth at 28 °C in A) low glucose (0.3% (w/v)) YPD media and B) in low glucose (0.3% (w/v)) YPD media with 1M sorbitol. Filled blocks indicate the percentage of strains with reduced viability. Open blocks represent the percentage of strains that have reduced viability and are zymolyase sensitive.



Cells generally grew better at optimum temperatures (28°C) when an osmotic stabiliser were added to the media. Mutants from the group with equal mannose and glucose levels (Group 2) and those with low glucose levels (Group 3), grew at a slightly improved rate while those with a low mannose content (Group 1)

grew significantly better. At a low temperature (14°C) there was not a great deal of difference between the growth of the three groups with or without osmotic stabiliser. At increased temperatures (37°C), under these experimental conditions, cell viability decreased with the addition of 1M sorbitol, particularly in those cells with a low β-glucan content (Group 3).

Reduced viability of cells with an osmotic stabiliser could be attributed to several biochemical mutations that reduce sensitivity to high osmolarity (Hirayama *et al.*, 1995). A mutation in one of the several *HOR* genes responsible for the synthesis of glycerol, an important osmoprotectant, can lead to cell lysis (Hirayama et al., 1995). In addition, a mutation which interferes with the functioning of the HOG pathway can also lead to cell lysis and loss of viability in high osmolarity media.

The stationary phase phenotype of *cwh47* is reversed by the addition of an osmotic stabiliser. Microscopic observations, undertaken in this study, revealed that these cells were multi-budded in low osmolarity but this condition was corrected by the addition of sorbitol. This suggests that this mutation is osmotically sensitive. *CWH47* encodes the *PTC1* gene product, a serine-threonine protein phosphatase which has a regulatory function in the *HOG1* signal transduction pathway. Disruption of the *CWH47* gene leads to a 25 % reduction of β1,6-glucan (Ram, 1996). This could also lead to reduced binding sites for proteins. Future experiments could investigate whether culturing the cells in a high osmolarity reverses killer toxin resistance.

PTC1 is an opposing regulator in low osmotic conditions. This cell wall defect probably does not occur in high osmotic conditions because the pathway

switches to the other regulator, *PBS2*. Over-expression of *PBS2* also leads to a reduction in β1,6-glucan levels which indicates that *pbs2* may also regulate genes involved in cell wall synthesis (Ram, 1996). When *PTC1* is expressed, permeability of the cell wall may increase, subsequently levels of glycerol would slowly reduce. Over-expression of *PBS2* leads to a 40% increase in glycerol content and disruption in *PTC1* leads to a 50% increase in glycerol content (Jiang *et al.*, 1995). The elevated glycerol content caused by *ptc1Δ* was suppressed by disruption in *PBS2* while a *pbs2Δ ptcΔ* double mutant contained wild type levels of glycerol (Jiang *et al.*, 1995). The over-expression of *PBS2* leads to a reduction in β1,6-glucan. Over-expression of *PBS2* or disruption of the *PTC1* gene leads to increased levels of exo-β-glucanase activity, an enzyme involved in cell wall synthesis, while disruption in *PBS2* leads to decreased levels of exo-β-glucanase activity (Santos *et al.*, 1979; Jiang *et al.*, 1995; Ram, 1996). The *cwh47* phenotype has a reduced ratio of glucose to mannose. This supports the theory that regulation of *PBS2* and *PTC1* is directly involved in cell wall synthesis.

CWH41 encodes a novel endoplasmic reticulum membrane *N*-glycoprotein involved in β1,6-glucan assembly. Disruption in this gene leads to a 50% reduction in cell wall β1,6-glucan. Severe β1,6-glucan defects lead to the secretion of cell wall glucomannoproteins. As an osmotic stabiliser does not correct the defect and *cwh41* is not Zymolyase resistant, this suggests that permeability of the cell wall is relatively unaffected by this defect. Studies in DEAE-dextran sensitivity seem to confirm that this strain does not have high cell wall permeability (Table 3.5.1). This may imply that a defect in the cell membrane is also contributing to loss of viability.

3.5 Further characterisation of mutants with reduced viability in stationary phase.

A number of mutants (20) were selected for further analysis and subjected to further tests such as measuring cell wall porosity (Relative DEAE-dextran sensitivity). This method determines the amount of cell leakage induced by polycations interacting with the cell surface. The ratio between DEAE-dextran (large hydrodynamic radius, 500 kDa) and poly-L-lysine (small hydrodynamic radius, 50 kDa) induced leakage of UV absorbing compounds is measured to estimate cell wall porosity.

In *cwh26* and *cwh46-2* the stationary phase defect was not corrected by the addition of sorbitol to the media. *cwh46-2* has increased cell wall permeability according to the assay for DEAE-dextran sensitivity (Table 3.3.1). Therefore other factors besides a more permeable cell wall may be contributing to the stationary phase phenotype. They both also share Papulacandin B hypersensitivity (Ram *et al.*, 1994). Papulacandin B is an antibiotic that inhibits β1,3-glucan synthesis. Eleven of the *CWH* mutants showed a greater sensitivity to papulacandin B (Ram *et al.*, 1994). Six of these strains also have reduced viability in stationary phase (*cwh3, cwh18, cwh26, cwh37, cwh46-2* and *cwh53-1*). *Cwh3* is hypersensitive to Zymolyase and papulacandin B but the defect is corrected with 1M sorbitol. The contributing factor then may be caffeine sensitivity which *cwh26* and *cwh46-2* both share. Caffeine sensitivity indicates a defect in signal transduction therefore the loss of ability to respond to stationary phase and extreme environments could be the predominant factor in loss of viability in these two strains.

Strain:	Cells/ml Exp Phase 28°C † (*n*= 6)	††Relative DEAE Dextran sensitivity	Time of reduced viability (weeks). Incubation temperature. 14°C	28°C No sorbitol	37°C	14°C	28°C + sorbitol	37°C	Phenotype‡
53-1**a**	$6.90x10^6$	32.11	3	5	5	3	>5	>5	Papulacandin B-hs, NaCl hs.
53-1α	$1.00x10^7$	27.31	>5	>5	>5	3	>5	>5	"
52-1**a**	$6.69x10^6$	39.87	>5	5	5	3	>5	>5	
52-1α	$4.07x10^5$	33.00	>5	5	5	3	>5	>5	
47**a**	$7.39x10^6$	--	>5	5	>5	>5	3	>5	Aggregating cells, killer resistant.
47α	$5.48x10^6$	--	>5	>5	5	3	>5	>5	"
46-2**a**	$4.24x10^5$	41.98	3	>5	5	3	5	5	High cell permeability, papulacandin B-hs, Rho$^-$,
46-2α	$1.47x10^6$	36.98	>5	>5	5	>5	>5	5	Zymolyase-hs, caffeine-hs.
44-1**a**	$1.14x10^7$	--	5	>5	5	5	>5	3	Aggregating cells.
42α	$9.79x10^6$	--	>5	>5	5	>5	>5	>5	Zymolyase-hs, NaCl hs.
41**a**	$5.74x10^6$	31.66	>5	>5	5	>5	>5	3	Killer-resistant.
41α	$5.90x10^6$	27.83	>5	>5	5	>5	>5	3	"
37**a**	$2.15x10^6$	32.98	5	>5	5	>5	>5	3	Papulacandin B-hs.
36**a**	$9.32x10^4$	--	5	3	5	3	3	3	Slow growth, NaCl-hs.
36α	$3.11x10^6$	25.90	5	>5	3	3	3	3	
28α	$6.47x10^6$	22.83	>5	5	5	3	>5	>5	
26α	$2.91x10^6$	--	>5	>5	3	>5	>5	3	Papulacandin B-hs, Zymolyase-hs, caffeine-hs.
25**a**	$2.59x10^6$	--	>5	>5	5	3	>5	3	
25α	$1.69x10^6$	26.42	>5	>5	5	3	>5	3	
23**a**	$3.17x10^6$	--	>5	>5	5	>5	>5	3	
19**a**	$6.20x10^6$	--	>5	>5	3	>5	>5	>5	Caffeine-hs.
19α	$5.32x10^6$	--	>5	>5	3	5	5	3	"
18**a**	$3.15x10^6$	--	>5	>5	>5	>5	>5	>5	Papulacandin B-hs, Rho$^-$.
18α	$2.40x10^5$	--	>5	>5	5	3	5	5	"
17**a**	$4.34x10^6$	49.44	>5	>5	5	3	5	5	High cell permeability, caffeine-hs.
17α	$5.08x10^6$	45.15	>5	>5	3	>5	>5	>5	"
13**a**	$2.79x10^5$	--	>5	>5	5	>5	>5	>5	Killer resistant, Zymolyase-hs.
10α	$2.00x10^7$	--	>5	>5	5	>5	>5	>5	Zymolyase-hs.
5**a**	$1.14x10^7$	--	3	>5	5	>5	>5	>5	Zymolyase-hs.
5α	$1.01x10^7$	--	>5	>5	>5	>5	>5	>5	"
3**a**	$2.20x10^6$	--	>5	>5	5	>5	>5	>5	Aggregating cells, Papulacandin B-hs, Zymolyase-hs.
3α	$7.10x10^6$	--	5	3	5	>5	3	>5	"

† Cells were first grown in liquid YPD (0.3% (w/v) glucose) then transferred to solid medium and incubated at the specified temperature.

†† Preliminary results based on one experiment, cells were grown for 2 weeks. -- not determined.

‡ Phenotypic tests involving Zymolyase, Papulacandin B, Caffeine, K1 killer toxin, temperature sensitivity and Rho from Ram *et al* (1994). NaCl sensitivity from Naik (1998).

Recent studies on the *WSC* genes, *WSC1*, *WSC2* and *WSC3,* have revealed that cells with a disruption in one of these genes show cell lysis at 37°C that is rescued with an osmotic stabiliser (Verna *et al.*, 1997). The *wscΔ* phenotype is also rescued by overexpressing *RHO1* or *PKC1* which implies they are connected to the PKC1-MPK1 pathway. Caffeine sensitivity is associated with the PKC1-MPK1 signal transduction pathway and several mutants involved in growth control such as *bcy1* and *ard1* are caffeine sensitive. These mutants also have a weakened cell wall but were not found to be Calcofluor White hypersensitive.

Four of the CWH strains (*cwh4*, *cwh6*, *cwh7* and *cwh50*) are already known to have cell lysis defects at 37 °C which is corrected by the addition of sorbitol but not by overexpressing *PKC1* (Ram, 1996). This study also found that these strains exhibit this phenotype, which was also pronounced in *cwh4* and *cwh7* at 28 °C. Starvation may increase sensitivity to 37 °C to the selected group with the stationary phase phenotype. The heat shock sensitivity of *wsc* mutants can be corrected by the inactivation of the RAS-cAMP pathway (Verna *et al.*, 1997). The *WSC* genes are also nutritionally sensitive. Deletion of all three genes results in the inability of the strain to grow on YPD but they remain viable on SC medium. Mutations in the genes of other cell wall proteins give similar phenotypes. *SED1* responds to a weakened cell wall or temperature shock (Caro *et al.*, 1997). *CWP1* transcripts and protein levels are induced in cells with weakened cell wall and cells with a reduction in cell wall protein incorporation have increased permeability and are hypersensitive to Zymolyase (Ram, 1996; Van Der Vaart *et al.*, 1995).

The five *K1* killer toxin resistant strains (*cwh13/erd1*, *cwh30/kic1*, *cwh41/gls1*, *cwh47/ptc1*, *cwh48/kre6*) were selected to assess stress and stationary phase characteristics in yeast within this study. This is because they have previously characterised genes and are known to have mutations that vary all cell wall components. For instance they differ in levels of β1,6-glucan, β1,3-glucan, chitin and mannoproteins. They also have mutations that effect different pathways. *CWH13* is involved in the secretory pathway while *PTC1* is a component of an osmoregulatory pathway. Studying these strains may enable a critical analysis of how the cell wall contributes to stress and stationary phase integrity.

3.6 Summary

Cell wall composition contributes to the loss of cell viability in stationary phase under certain conditions. Strains with low cell wall mannose content are more inclined to lose viability in early stationary phase. In many instances this can be corrected by the addition of an osmotic stabiliser. Deviation from the optimum growth temperature also led to reduced viability in specific strains. At high temperatures loss of viability is greatest in cells with reduced mannose content. This emphasises the role played by cell wall proteins in protecting the cell from environmental change.

Viability in strains with an additional phenotype, as well as Calcofluor-White sensitivity, is also adversely influenced by environmental conditions. Strains which are Zymolyase-hypersensitive lose viability at low temperatures and in high osmolarity at increased temperatures. Papulacandin B-sensitive strains also

give reduced viability in temperatures that deviate from optimum growth conditions. Caffeine-sensitive strains are mainly restricted by high temperatures while Rho$^-$ strains are most affected by changes in osmolarity. *K1* killer toxin resistant (*kre*) strains showed loss of viability in response to a range of different environments and gave reduced viability in optimum conditions. This indicates that the nature of the mutation in *kre* strains affects phenotype in several different ways. It was also the only phenotype in this study that did not have the highest viability at optimum temperatures. As the cell wall composition of the *kre* strains has been well studied because of their involvement in β1,6-glucan synthesis and its subsequent association with GPI proteins (Meaden *et al.*, 1990; Brown *et al.*, 1993; Brown and Bussey, 1993; Roemer *et al.*, 1994), these strains were selected to continue assessing the nature and influence of cell wall composition in stationary phase.

Chapter 4

Stationary phase characteristics in *kre* strains.

K1 killer toxin is produced by Killer strains of *S. cerevisiae* which contain double-stranded RNA viruses that secrete protein toxins fatal to virus-free cells. The toxin acts on sensitive yeast cells to perturb potassium homeostasis which eventually leads to cell death (Ahmed *et al.*, 1999). The *K1* killer toxin molecule is a small protein that binds to a β1,6-glucan receptor to form a lethal cation channel across the plasma membrane. Yeast strains that have reduced levels of β1,6-glucan in the cell wall are often resistant to *K1* killer toxin (Table 4.0.1). Strains that are resistant to *K1* killer toxin (*kre* strains) have been used extensively to study the incorporation of β1,6-glucan into the cell wall (Meaden *et al.*, 1990; Brown *et al.*, 1993; Brown and Bussey, 1993; Roemer *et al.*, 1994). By studying a number of *kre* mutants the pathway in which β1,6-glucan is constructed has been elucidated. *KRE* gene products and their functional homologues have been localised in the ER [*KRE5* (Meaden *et al.*, 1990)], Golgi [*ERD1*, *KRE6*, and *SKN1* (Roemer *et al.*, 1994)], the cytoplasm [*KRE1* (Brown *et al.*, 1993)] and the cell surface [*KRE9* (Brown and Bussey,1993)].

Overall, the information so far available, implies that synthesis of β1,6-glucan follows the secretory pathway and is then completed at the cell surface but the exact biochemical function of the majority of these gene products is largely unknown (Shahinian *et al.*, 1998). Recently, it has been proposed that β1,6-glucan synthesis takes place at the cell surface while the *KRE* genes are involved

Table 4.0.1. Genes known to indirectly confer *K1* Killer toxin resistance *in S. cerevisiae*.

ORF	Gene	Gene product/function	Localisation	References
YAL023c	*PMT2*	Mannosyltransferase	ER membrane, integral membrane	Gentzsch and Tanner (1996)
YBL082c	*RHK1*	Mannosyltransferase	ER membrane, integral membrane	Kimura *et al* (1997)
YBR023c	*CHS3*	Chitin synthase III	Plasma membrane (cell cycle dependant)	Kollar *et al* (1997)
YDL006w	*PTC1*	Protein ser/thr phosphatase 2c	Unknown	Ram *et al* (1994)
YDL095w	*PMT1*	Mannosyltransferase	ER membrane, integral membrane	Gentzsch and Tanner (1996)
YDR483w	*KRE2*	α-1,2-mannosyltransferase	Golgi, integral membrane	Gentzsch and Tanner (1996)
YGL027c	*CWH41*	ER glucosidase I	ER, integral membrane	Ram *et al* (1994)
YGR166w	*KRE11*	β-glucan synthesis protein	Cytoplasm	Brown and Bussey (1993)
YGR229c	*SMI1*	β-1,3-glucan synthesis protein	Cytoplasm	Hong *et al* (1994)
YHR102w	*KIC1*	Ser/thr protein kinase	Unknown	Ram *et al* (1994), Sullivan *et al* (1998)
YHR187w	*IKI1*	Confers sensitivity to killer toxin	Unknown	Yajima *et al* (1997)
YIL154c	(*IMP2*)	Sugar utilization regulatory protein	Mitochondrial, Mitochondrial inner membrane	Nunnari *et al* (1993)
YKL110c	*KTI12*	Involved in killer toxin resistance	Unknown	Butler *et al* (1994)
YKR061w	*KTR2*	Mannosyltransferase	Golgi, integral membrane	Gentzsch and Tanner (1996)
YLR384c	*IKI3*	Confers sensitivity to killer toxin	Nuclear, DNA associated	Yajima *et al* (1997)
YNL219c	*ALG9*	Mannosyltransferase	ER	Gentzsch and Tanner (1996)
YNL322c	*KRE1*	Cell wall protein	Plasma membrane, cell wall	Kollar *et al* (1997)
YNL329c	*PEX6*	Peroxisomal assembly protein	Cytoplasm	Distel *et al* (1996)
YOR099w	*KTR1*	Mannosyltransferase	Golgi, integral membrane	Gentzsch and Tanner (1996)
YOR336w	*KRE5*	Killer toxin-resistance protein	ER (soluble)	Kollar *et al* (1997)
YPR159w	*KRE6*	Glucan synthase subunit	Golgi, integral membrane	Ram *et al* (1994)

in the synthesis of an acceptor structure for later β1,6-glucan addition (Montijn *et al.*, 1998). In this study a number of *kre* mutants with reduced levels of β1,6-glucan were used to study how reduced β1,6-glucan synthesis and cell wall defects influence stationary phase characteristics, such as increased cell wall protein content and accumulation of intracellular trehalose.

4.1 Phenotypes in *kre* mutations and the effect on β1,6-glucan assembly.

The direct effect *KRE* mutations have on certain cell processes indirectly affects functions in other areas of the cell such as in cell wall construction and cell metabolism. The number of *kre* strains that have so far been isolated serve to emphasise the complex interactions needed to produce the cell wall and the processes involved in β1,6-glucan synthesis (Table 4.0.1). Mutations that are known to cause loss of viability in stationary phase also occur at different functional levels. This study assesses the influence of *kre* mutations on cell viability in stationary phase and resistance to stress.

The five *kre* strains used in this study all have distinct phenotypes caused by mutations in different regions of the genome (Table 4.1.1.). *CWH13/ERD1* is involved in the retention of ER proteins (Hardwick *et al.*, 1990). *CWH30/KIC1* and *CWH47/PTC1* have mutations which affect cell cycle regulation and metabolism (Sullivan *et al.*, 1998; Jiang *et al.*, 1995). *CWH41/GLS1* is involved in *N*-glycosylation (Romero *et al.*, 1997) and *CWH48/KRE6* is required for cell wall construction (Roemer and Bussey, 1991), possibly as a glycotransferase.

Table 4.1.1. Mutations and carbohydrate composition in the cell walls of *kre* strains used in this study (Ram *et al.*, 1994).

Strain	Gene	Gene product	Cell wall composition %			
			Chitin	Glucose	Mannose	Reduction in β1,6 glucan
cwh13	*ERD1*	Required for retention of ER proteins.	3.6	59.5	36.9	unknown
cwh30	*KIC1*	Serine/threonine protein kinase.	1.4	50.7	48.9	"
cwh41	*GLS1*	Glucosidase I.	1.2	45.0	53.8	50%
cwh47	*PTC1*	Protein serine/threonine phosphatase 2c.	1.1	41.3	57.4	25%
cwh48	*KRE6*	Glucan synthase subunit.	2.2	38.7	59.1	50%

The *kre* strain with a low mannose content *cwh13* has a mutation that affects *ERD1*, a gene required for the retention of luminal ER proteins. The exact function of Erd1p is unknown but it is believed to be a membrane protein, with several transmembrane domains, that contributes to protein modification in the Golgi sorting stage of the secretory pathway (Hardwick *et al.*, 1990). Strains that contain an *ERD1* disruption are known to have defects in the maturation of glycoproteins (Hardwick *et al.*, 1990). *CWH41* is also involved in the modification of proteins. It has recently been discovered that Cwh41p is the enzyme glucosidase I (Romero *et al.*, 1997). Glucosidase I is responsible for the first step of glucosyl-*N*-chain processing. It is now believed that β1,6-glucan is attached to protein *N*-chains within the cell wall matrix (Shahinian *et al.*, 1998). This could explain the low level of β1,6-glucan in this strain. A strain used to study stationary phase in *S. cerevisiae ard1* also has a mutation which affects protein modification (Whiteway and Szostak, 1985). The *ARD1* gene product is a subunit of a protein amino-terminal acetyltransferase which is involved in protein *N*-terminal acetylation. Mutants of *ARD1* do not recognise starvation signals and

cease to proliferate without acquiring stationary phase characteristics (Whiteway and Szostak, 1985).

New evidence is emerging that although β1,6-glucan is involved in the attachment of GPI proteins to the cell wall, it is not the only factor involved (Shahinian *et al.*, 1998; Kapteyn *et al.*, 1996; Kollar *et al.*, 1997). Several studies involving α-agglutinin have shown that protein attachment varies in different *kre* mutants. Secretion of α-agglutinin into the surrounding medium increases in *kre1, kre5, kre6, kre9* and *kre11* (Lu *et al.*, 1995). Recently levels of α-agglutinin in *cwh41* have been found to be unaffected despite substantially reduced β1,6-glucan levels in the cell wall (Shahinian *et al.*, 1998). The *cwh48/kre6* strain assessed in this study is known to lose the ability to attach cell wall proteins. *KRE6* encodes a β-glucan synthase required for the synthesis of major β-glucans in the yeast cell wall (Roemer and Bussey, 1991). Strains with mutations in this gene have reduced levels of β1,3-glucan, β1,6-glucan and GPI anchored proteins. These findings suggest that levels of stress related proteins such as Sed1p could also be reduced and could contribute to loss of viability. Further investigations made within this study support this theory (Chapter 5).

Some phenotypes induced by *KRE* mutations lead to morphological and cell cycle defects. *cwh47* has a mutation which affects the *PTC1* gene. As previously described (Chapter 3, 3.4), the *PTC1* gene product is a serine threonine phosphatase which regulates a genetic pathway that interconnects cell wall construction with osmotic control (Schuller *et al.*, 1994; Ram *et al.*, 1994). *PTC1* is involved in a two component osmoregulatory system. *PTC1* regulates in low

osmotic conditions. In high osmotic conditions the pathway switches to the other regulator, *PBS2*.

Another *kre* strain with a mutation that leads to cell division and morphological defects is *cwh30*. *cwh30* contains a mutation which disrupts *KIC1*, an essential gene which encodes a serine threonine protein kinase that interacts with a cell cycle regulator, Cdc31p. This mutant has also been found to contain high glycerol levels believed to be caused by the up-regulation of the HOG pathway. Low levels of β1,6-glucan could be caused by increased activity of this pathway in a similar way that over-expressing *PBS2* increases glycerol content and reduces levels of β1,6-glucan. Up-regulating the HOG pathway by overexpressing *PBS2* is thought to increase exo-β-glucanase activity which in turn leads to a reduction in cell wall β1,6-glucan (Santos *et al.*, 1979; Jiang *et al.*, 1995; Ram, 1996).

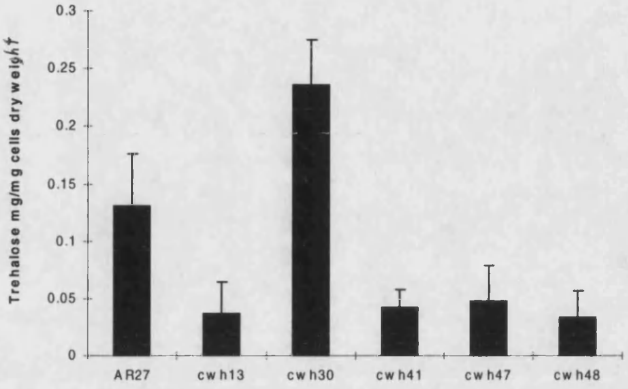
Results and Discussion.

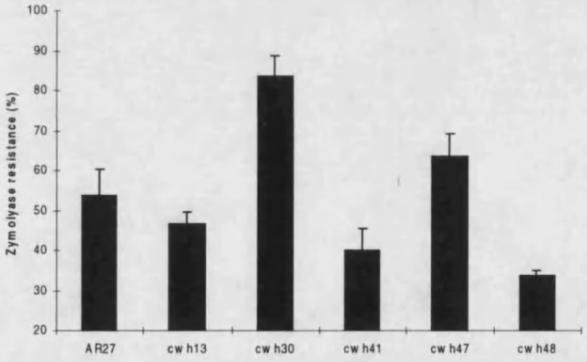
4.2 Influence of cell wall structure on stress characteristics in *kre* strains.

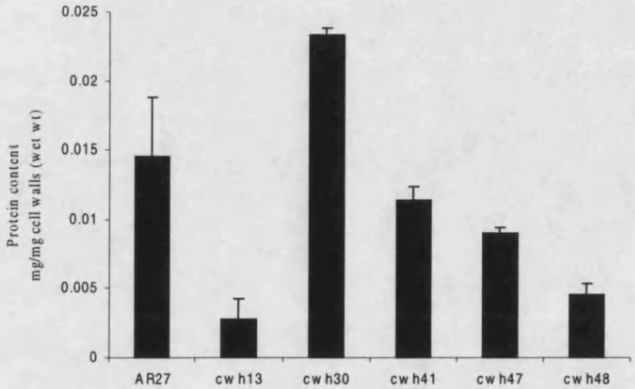
4.2.1 Intracellular trehalose levels.

Trehalose levels were measured enzymatically with acid-trehalase by a technique described by Kienle *et al.* (1993). All measurements were assessed in relation to cell dry weight. There is a significant rise in trehalose levels in *cwh30/kic1* when compared to the wildtype cells AR27 (Fig. 4.2.1). All other *kre* strains have a significantly reduced intracellular trehalose content when compared to AR27. After two weeks growth AR27 has a trehalose level of 0.131 mg/mg cell dry

Figure 4.2.1 Stress characteristics in *kre* strains of *S. cerevisiae*. Intracellular trehalose content, zymolyase resistance and cell wall protein content after two weeks growth in liquid YPD (2% (w/v) glucose) with constant shaking at 28°C (Mean ± SEM).







weight. The lowest intracellular trehalose content was found in strains with the greatest sensitivity to Zymolyase, *cwh13/erd1* (0.036 mg/mg) and *cwh48/kre6* (0.033 mg/mg). The highest trehalose level was found in the strain with the greatest resistance to Zymolyase *cwh30/kic1* (0.235 mg/mg).

Yeast cells accumulate the storage carbohydrates glycogen and trehalose when entering stationary phase. Glycogen accumulation peaks at the diauxic shift just before glucose exhaustion, whereas trehalose levels begin to rise at the diauxic shift and continue to accumulate until cells reach stationary phase. When stationary phase is reached trehalose levels begin to fall (Lillie and Pringle, 1980). In view of these facts, this study measured trehalose levels after two weeks. By then wild type cells would either be in a post-diauxic or early stationary phase stage of growth. Glycogen and trehalose are thought to have two different functions during starvation in yeast. Glycogen is thought to act as a reserve carbohydrate that is metabolised on glucose limitation while trehalose is thought to function in general stress resistance (De Winde *et al.*, 1997). There is no evidence that viability increases with intracellular accumulation of these carbohydrates although there may be a correlation between stress resistance and intracellular trehalose levels (Attfield *et al.*, 1992).

In this study, the increase in trehalose levels of *cwh30/kic1* could either be due to increased trehalose biosynthesis or decreased trehalase activity. When *S. cerevisiae* is subjected to stress, trehalase activity decreases as cells adopt stress characteristics. The defect in the cell wall of *cwh30/kic1* could be inducing stress characteristics when grown under experimental conditions. However, these

results demonstrate that other factors besides a defective cell wall must be contributing to the rise in trehalose as although the other *kre* strains have defective cell walls they have a reduced level of intracellular trehalose. It could be possible that *cwh30/kic1* is adopting a different metabolic pattern to the other *kre* strains and is unable to grow on a fermentable carbon source such as glucose. The characteristics of *cwh30/kic1* are assessed further in sections 4.3 and 4.4.

4.2.2 Zymolyase sensitivity.

Zymolyase resistance was assessed in whole cells by a method described by Ram *et al.* (1994). Resistance to degradation was measured by spectrometry as the difference in percentage of absorbance at 520 nm after incubating yeast cells with Zymolyase 20T. Samples from yeast cell cultures were measured at intervals of 7 days in each strain over a 5 week period. Strains were incubated at 28°C in flasks containing liquid YPD (0.3% (w/v) glucose) with and without 1M sorbitol. Results are an average measurement from 3 separate experiments.

After 2 week's growth, the wild type strain AR27 had a Zymolyase resistance of 54% (Fig.4.2.1). The strain that had the highest trehalose content, *cwh30/kic1*, also had the greatest Zymolyase resistance (84%). By contrast, the strain with the lowest trehalose level after 2 week's growth, *cwh48/kre6* had the lowest resistance to zymolyase (34%). The Zymolyase resistance in *cwh30/kic1* has also been detected by other researchers (Sullivan *et al.*, 1998; Vossen J and Klis F, unpublished data). Lack of permeability in the cell wall of these mutants could account for these observations. In electron micrographs it is possible to see that the cwh30/*kic1* mutation elevates levels of electron dense mannans in the cell

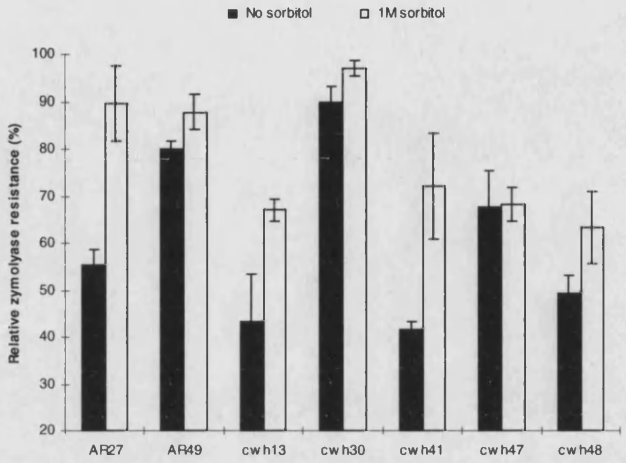
wall. In the *kre* strains studied here, there seems to be a correlation between Zymolyase sensitivity and intracellular trehalose content.

Zymolyase resistance was also assessed in strains that were grown with an osmotic stabiliser (1M sorbitol) added to the media (Fig.4.2.2). Zymolyase resistance increased in all strains grown with 1M sorbitol. This increase was significant after one week's growth in all of the strains apart from *cwh30/kic1* and *cwh47/ptc1*. Zymolyase resistance in *cwh30/kic1* was relatively unaltered when 1M sorbitol was added to the media whereas under the same conditions in the wild type strain, AR27, Zymolyase resistance increased. This indicates that cell wall structure is regulated during normal growth in changing conditions. Stress characteristics in the *cwh30/kic1* strain appear to be permanently induced therefore increasing osmolarity in this strain would have no noticeable affect on Zymolyase resistance.

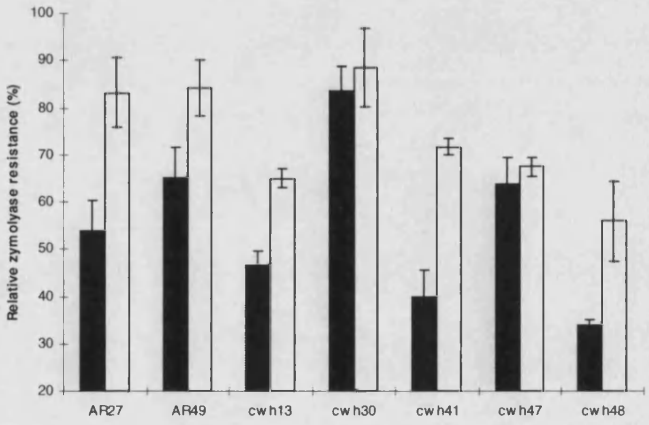
Within the *kre* strains, *cwh48/kre6* had the greatest sensitivity to Zymolyase (Fig. 4.2.3). *cwh13/erd1* was initially sensitive but showed increased resistance to Zymolyase as the culture entered further into stationary phase. This strain has a low mannose cell wall content and a mutation that secretes proteins into the surrounding media (Hardwick *et al.*, 1990). Increased resistance to Zymolyase in this strain could be due to changes in cell wall composition and an induction of protective cell proteins, such as Sed1p, upon entry into stationary phase. In strains with stationary phase phenotypes *bcy1-1*, *ard1* and *ubi4* there is sensitivity to Zymolyase during exponential phase but resistance increases as the culture progresses through to stationary phase (Fig. 4.2.3).

Figure 4.2.2. Zymolyase resistance in *kre* yeast strains grown with and without an osmotic stabiliser (1M sorbitol). Cultures were grown for 1 week, 2 weeks and 5 weeks in liquid YPD (2% (w/v) glucose). Mean ± SEM.

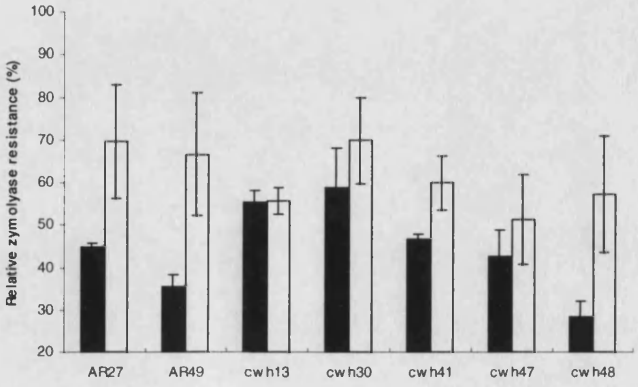
a) 1 week.



b) 2 weeks.



c) 5 weeks.



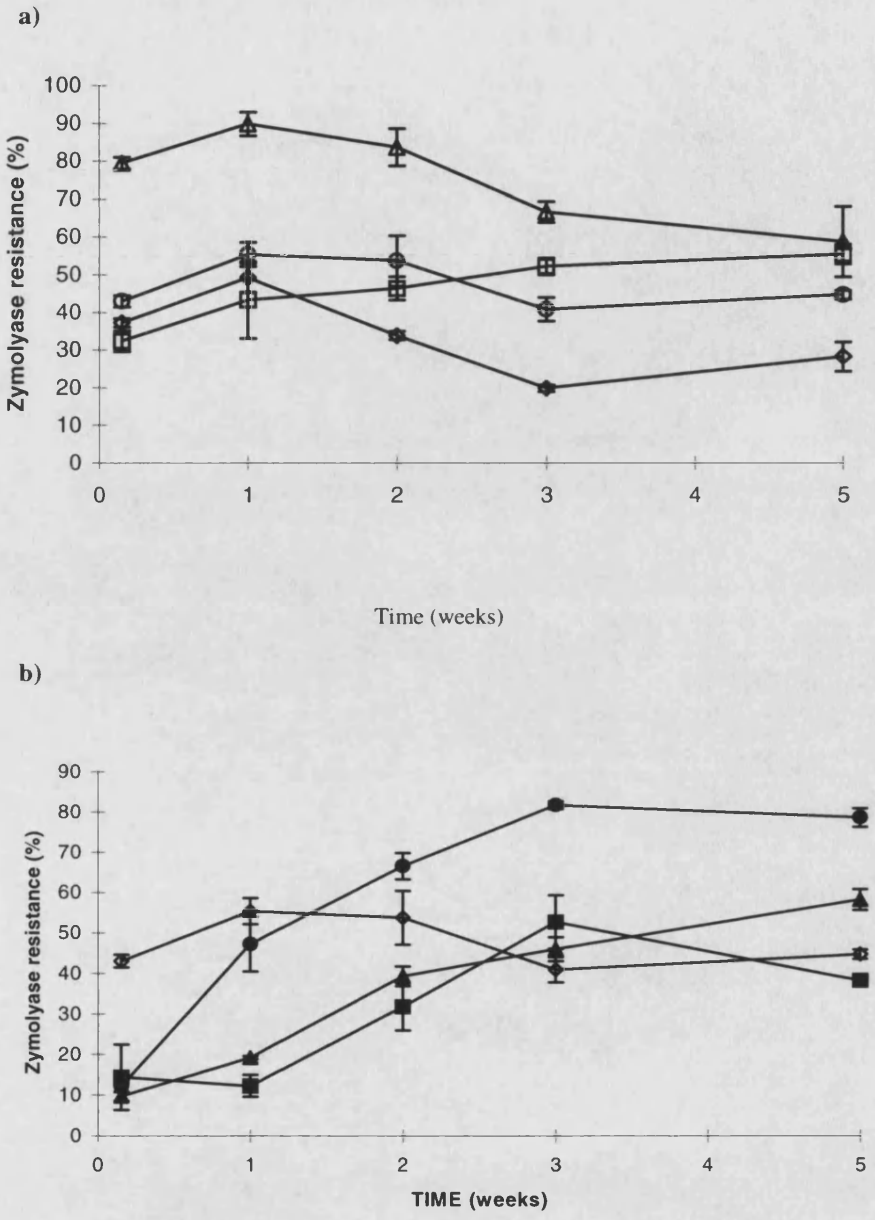


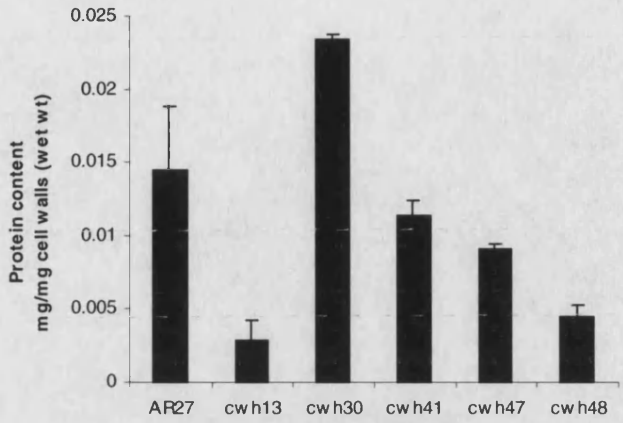
Figure 4.2.3. Zymolyase resistance in strains grown in YPD with 0.3 % (w/v) glucose from exponentional growth to long term stationary phase (5 weeks). a) Zymolyase resistance in *kre* strains AR27 (○), *cwh13* (□), *cwh30* (Δ), *cwh 48* (◊). b) Zymolyase resistance in AR27 (◊) and strains that are known to lose viability in stationary phase; *bcy1* (●), *ard1* (■) and *ubi4* (▲). In each case results were obtained from three separate experiments. Mean ± SEM.

Overall, these results imply that certain cell wall defects make cells more susceptible to Zymolyase. This could be due either to reduced levels of protective mannoproteins or to a decrease in the Zymolyase binding site. In the case of *cwh13/erd1* a reduction in mannoproteins is the most likely cause. High resistance in *cwh30/kic1* could either be a consequence of increased mannoprotein levels in the cell wall or a change in the structure of the β-glucan matrix. The increased Zymolyase resistance in strains that are grown with 1M sorbitol added to the media implies that cell wall composition is altered in response to an increase in osmolarity. These changes are not as apparent in *cwh30/kic1* and *cwh47/ptc1* possibly because cell wall composition is not altered in response to changes in osmolarity.

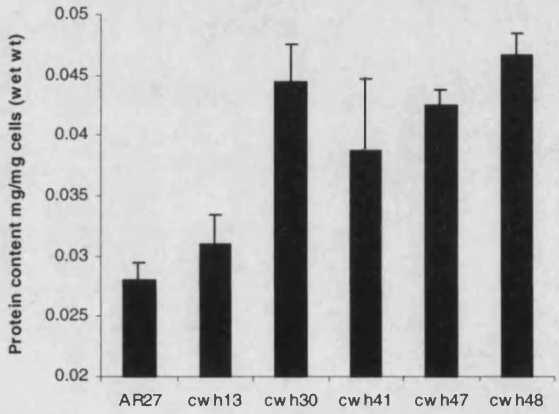
4.2.3 Cell wall protein levels.

Protein levels were measured in isolated cell walls by an assay described by Klis *et al.* (1998) using NaOH hydrolysis (Fig. 4.2.4). Laminarinase extracted cell wall proteins were also compared by SDS-PAGE (Fig. 4.2.5). Cell wall protein content was found to be reduced in four of the five *kre* strains (*cwh13/erd1, cwh41, cwh47/ptc1, cwh48/kre6*) when compared to the wild type strain AR27. In contrast, *cwh30/kic1* showed an increase in the quantity of cell wall proteins. The protein content of the media was also measured in these strains (Fig. 4.2.4). Only *cwh13/erd1, cwh41/gls11* and *cwh48/kre6* appeared to secrete proteins into the media. Protein levels in the media were virtually undetectable in the other strains (Fig. 4.2.4).

a)



b)



c)

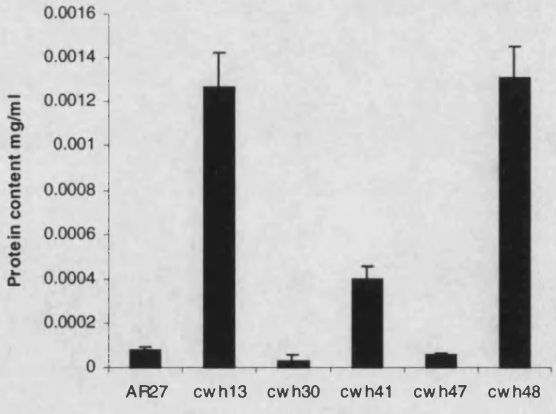


Figure 4.2.4. Protein levels of a) cell walls, b) intracellular extract and c) media in *kre* strains. Cell wall proteins were extracted by NaOH hydrolysis. Intracellular extracts were obtained by cell lysis with glass beads and media protein levels were obtained by desiccation. Bradford reagent was used to quantify protein level in each case. Mean ± SEM.

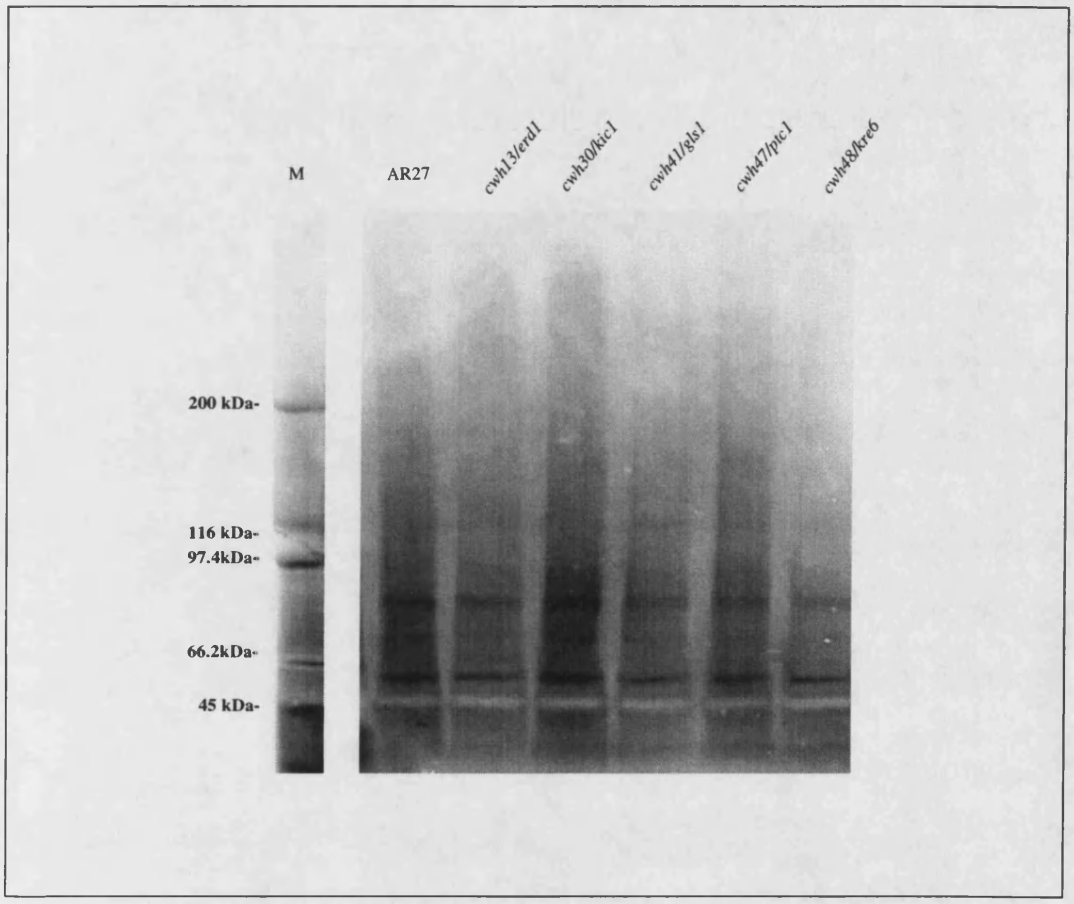


Figure 4.2.5. SDS-PAGE (3-11% gradient gel) analysis of laminarinase extracted cell wall proteins of *kre* strains. The acrylamide gel was stained with Coomassie Blue solution then counter stained with a diamide silver solution sensitive to glycoproteins. Electrophoresis illustrates that proteins extracted from cell walls of AR27, *cwh30/kic1* and *cwh47/ptc1* give denser staining than *cwh13/erd1*, *cwh41/gls1* and *cwh48/kre6*.

When cells approach stationary phase, they exhibit increased levels of disulphide bridges and *N*-glycosylation of mannoproteins (Sanz *et al.*, 1989). As in the other stress related characteristics, cell wall protein levels varied between the five *kre* strains. Again *cwh30/kic1* is adopting a stress response. Cell wall protein levels are higher in this strain then in the other *kre* strains. The strain with the lowest protein content *cwh48/kre6* is also the strain with the lowest Zymolyase resistance and trehalose levels. This strain and *cwh13/erd1* appear to be secreting a high amount of proteins into the media (Fig. 4.2.4). *ERD1* mutants are defective in the Golgi-dependent modification of glycoproteins. *KRE6* mutants are involved in β1,3-glucan synthesis. In *cwh13/erd1* Zymolyase resistance increases the further the strain enters into stationary phase. This could be due to the increase in glycosylation of stationary phase proteins. Increased glycosylation could prevent them from being secreted as freely into the media and allow them to become more readily incorporated into the cell wall matrix.

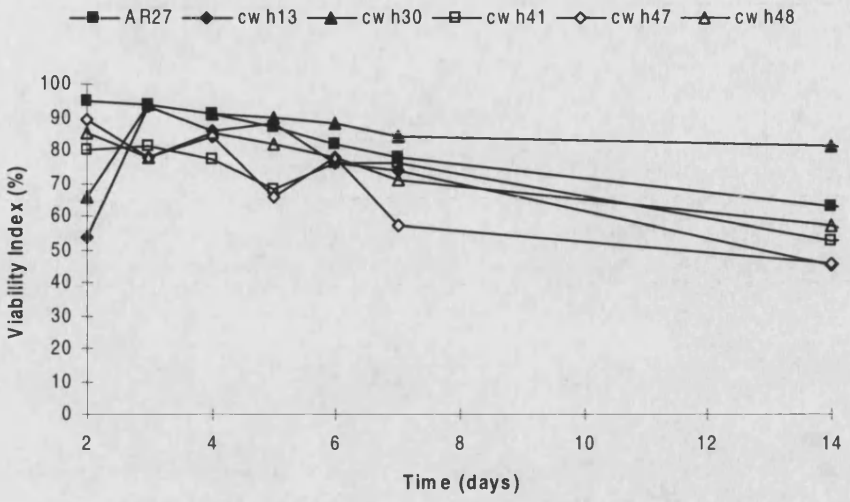
The *ERD2* deletion is suppressed by over-expressing a gene encoding a heavily glycosylated stationary phase protein Sed1p. Perhaps the mechanism by which heavily glycosylated proteins are incorporated into the cell wall can compensate for errors in protein modification that cause smaller proteins to be secreted into the media. Further investigations could be carried out in *cwh13/erd1* to assess whether cell wall protein levels and protein secretion vary in relation to growth phase. The low levels of proteins, Zymolyase resistance and trehalose in *cwh13/erd1* and *cwh48/kre6* imply that these strains have mutations that would impair their ability to survive starvation and other extreme environments.

4.2.4 Cell viability in stationary phase.

The cell viability of the *kre* strains was assessed in short term (14 days) and long term (6 months) stationary phase (Fig 4.2.6). In early stationary phase *cwh13/erd1* and *cwh47/ptc1* have the lowest number of cells/culture that have the ability to form colonies when transferred from liquid YPD (0.3% (w/v) glucose, 28°C) to solid YPD (2% (w/v) glucose, 28°C). The viability indices of *cwh13/erd1* and *cwh47/ptc1* are 45% and 45.5% respectively (Fig. 4.2.6 (a)). The wild type strain AR27 has a viability index of approximately 63% after 14 days under these growth conditions, whereas *cwh30/kic1* has the highest viability index of 81%. The viability of *cwh30/kic1* appears to increase in stationary phase although viability is reduced to 66% during exponential growth.

After 6 months incubation at 28°C in liquid YPD (2% (w/v) glucose) all the *kre* strains produce less colonies then the wild type strain, AR27 (Fig. 4.2.6(b)). *Cwh48/kre6* produced very few colonies after 6 months incubation at 28°C and was virtually non-viable (Fig. 4.2.6(b)). In this study *cwh48/kre6* was found to have the lowest viability in long term stationary phase. The other strains with low viability were *cwh47/ptc1* (~2,000 colonies/ml) and *cwh13/erd1* (~4,000 colonies/ml). The *kre* strains with the highest viability in long term stationary phase were *cwh30/kic1* and *cwh41/gls1* (6,000 – 8,000 colonies/ml).

a)



b)

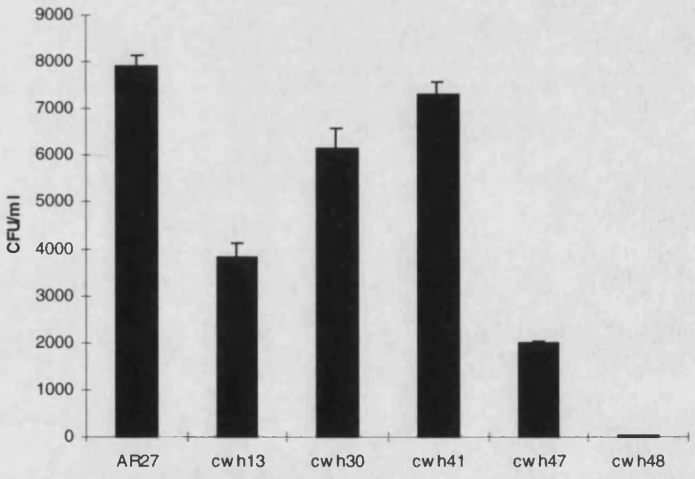


Figure 4.2.5. Cell viability in **a**) Short term (14 days) and **b**) long term (6 months) stationary phase *kre* strains. Viability Index was assessed by colony counts and cell numbers from 3 experiments. Long term stationary phase colony counts were the average of 4 readings from two separate cultures. Mean ± SEM.

4.3 Accumulation of trehalose in *kic1/cwh30* and other stress characteristics induced during optimal growth conditions.

Higher levels of cell wall proteins and Zymolyase resistance also accompany high intracellular trehalose levels in *cwh30/kic1* when compared to the parental strain AR27 (Fig. 4.2.1). Electron micrographs show that populations of *cwh30/kic1* cells take on a different morphology to normally dividing cells (Fig. 4.3.1). *cwh30/kic1* cells are rounder and have clearly visible nuclei when compared to AR27 cells. Cells appear to have adopted stress characteristics despite growing in optimal conditions.

Trehalose mobilisation may be required in exponentially dividing cells during fermentative growth. Several factors indicate that trehalose is mobilised prior to glycolysis. Yeast cells usually accumulate trehalose when the cell cycle has arrested or cell division and glycolysis is reduced. Trehalose levels increase in cells undergoing respiratory adaptation, in cells subjected to osmotic stress, cells under nutrient and carbon starvation, in heat stress, dehydration, pressure and in cells subjected to toxic chemical stress (Nwaka and Holzer, 1998). In contrast, the activity of trehalases increases upon the initiation of growth in stationary phase cultures. Addition of glucose to stationary phase cultures leads to rapid hydrolysis of trehalose via the Ras/cAMP pathway. Disruption of the *NTH1* gene leads to loss of neutral trehalose activity and an increase level of trehalose in dividing cells. The *NTH1* and *NTH2* genes have been found necessary for recovery of yeast cells following heat shock and when heat stressed cells are transferred to normal temperatures neutral trehalase activity increases (Nwaka and Holzer, 1998). Recovery of cells from heat shock is largely

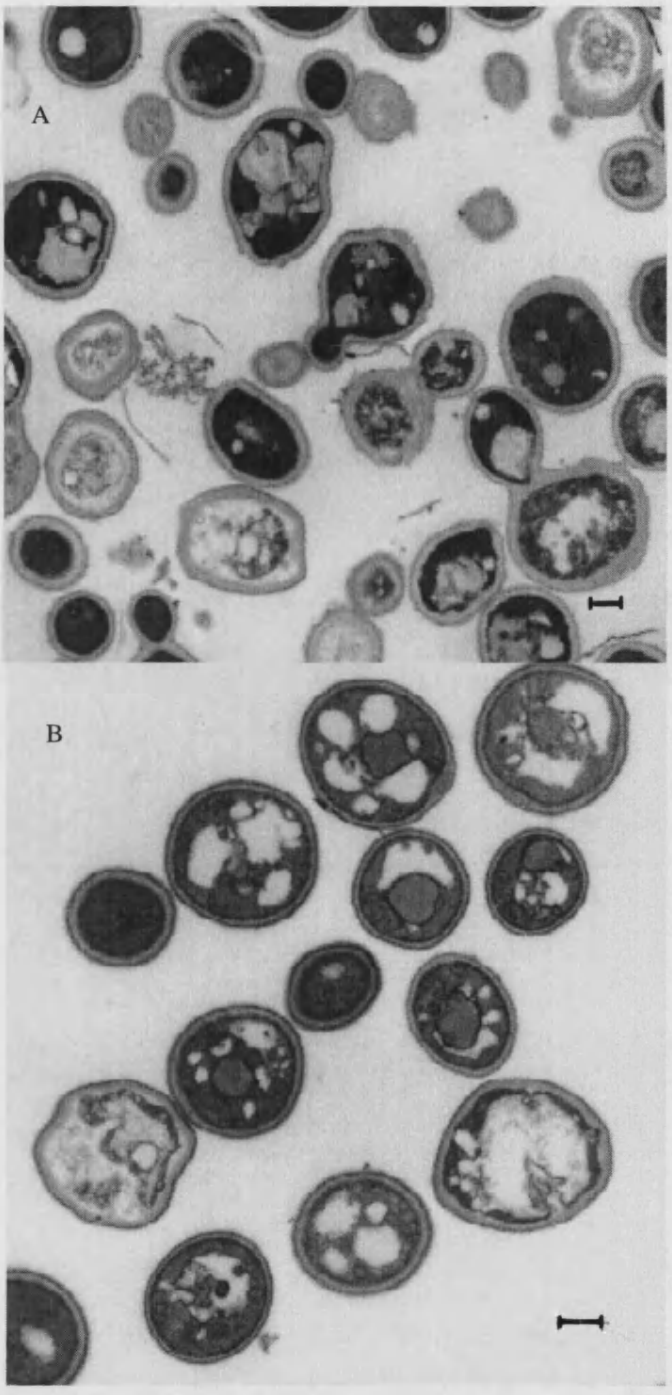


Figure 4.3.1. Transmission electron micrographs illustrating morphological differences between *S. cerevisiae* strains AR27 (A) and *cwh30/kic1* (B). Cells were grown in liquid YPD containing 2% (w/v) glucose for two weeks (28°C). Scale bar represents 1 μm.

attributed to neutral trehalase activity and not levels of trehalose (Nwaka and Holzer, 1998). Yeast cells that lack certain heat shock proteins have an increased sensitivity to heat but still accumulate trehalose (Nwaka and Holzer, 1998). Recent findings reveal that trehalose inhibits protein folding and aggregation (Singer and Lindquist, 1998). This could be the reason why trehalose has to be mobilised in cells that are performing rapid cell division. However, deletion of the *TPS1* gene encoding trehalose-6-phosphate synthase leads to the inability of cells to synthesise trehalose and prevents growth in glucose (Reinders *et al.*, 1997).

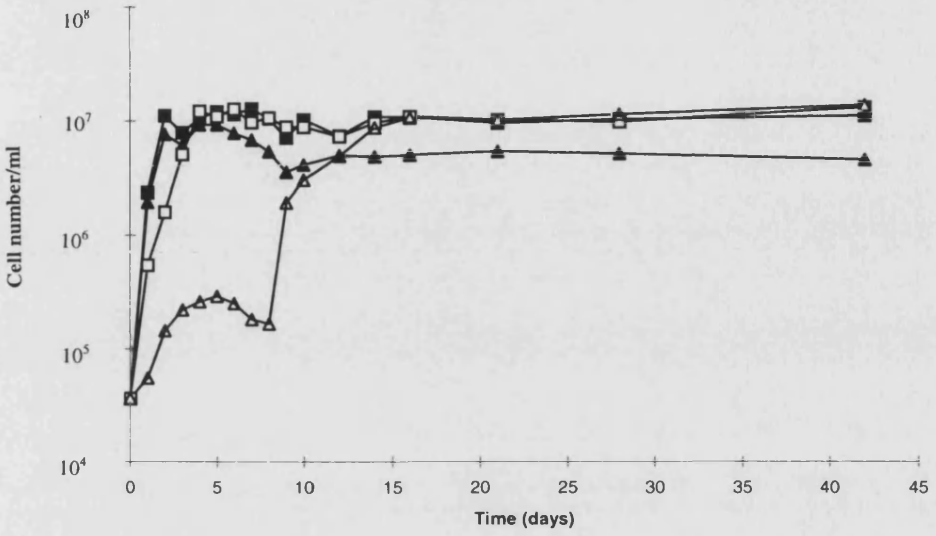
Several studies have correlated a rise in intracellular trehalose with a specific stage in physiological development. These steps also coincide with a temporary cessation of cell division, for instance, when cells are undergoing respiratory adaptation (Panek and Matton, 1977), entering stationary phase (Lillie and Pringle, 1980) or under various physical stresses such as changes in temperature (Kienle *et al*, 1993) or osmolarity (Singh and Norton, 1991). These factors have led to the assumption that elevated levels of trehalose act as a stress protectant. However, a number of studies have revealed that a high level of trehalose does not necessarily confer tolerance to some stress conditions. A mutant deficient in the synthesis of the heat shock protein Hsp104 was found to have less resistance to temperature stress than the wild type strain even though there was no significant difference in trehalose levels between the two strains (Winkler *et al*, 1991). Other studies have revealed that trehalose levels only appear to offer thermoprotection in yeast cells grown in a non-fermentable carbon source but not in cells grown on a fermentable carbon source (Van Dijck *et al.*, 1995).

Expression of the neutral trehalase genes, *NTH1* and *NTH2*, increases under various conditions such as in response to temperature change and toxic chemicals (Nwaka and Holzer, 1998). These genes are also necessary for recovery of yeast cells following heat shock. Deletion of trehalase genes affects growth on non-fermentable carbon sources. Trehalase mutants, *nth1* and *ath1*, have been found to grow poorly on glycerol when compared to a wild type strain (Nwaka *et al.*, 1995). Neutral trehalase is induced mainly in cells that are actively growing on glucose (Wiemken, 1990). The activity of neutral trehalase is reduced in yeast cells grown on a nonfermentable source such as glycerol. In conditions of high osmolarity, neutral trehalase activity is not increased (Zahringer *et al.*, 1997). Adaptation to high osmolarity, in yeast, is controlled by *HOG1* pathway (Schuller *et al.*, 1994; Ram *et al.*, 1994). In response to low osmolarity the MAP kinase pathway, *SLT2/MPK1,* is activated (Davenport *et al.*, 1995). Both pathways are thought to be involved in cell wall construction (Cid *et al.*, 1995).

4.4 Slow transition phase in *cwh30/kic1* cells when transferred to a non-fermentable carbon source.

To investigate further the resistance to Zymolyase and changes in trehalose levels in *cwh30/kic1,* cells were grown in either glucose or a non-fermentable carbon source. Although AR27 was found to grow slightly slower in exponential growth when compared to strains grown in glucose (Fig. 4.4.1(a)), it did not take as long to adapt to the media as *cwh30/kic1*. The lag phase for *cwh30/kic1* in glycerol was approximately eight days (Fig. 4.4.1(a)). Cells then recovered and reached the equivalent population size of AR27. *cwh30/kic1* grown in glucose had the

a)



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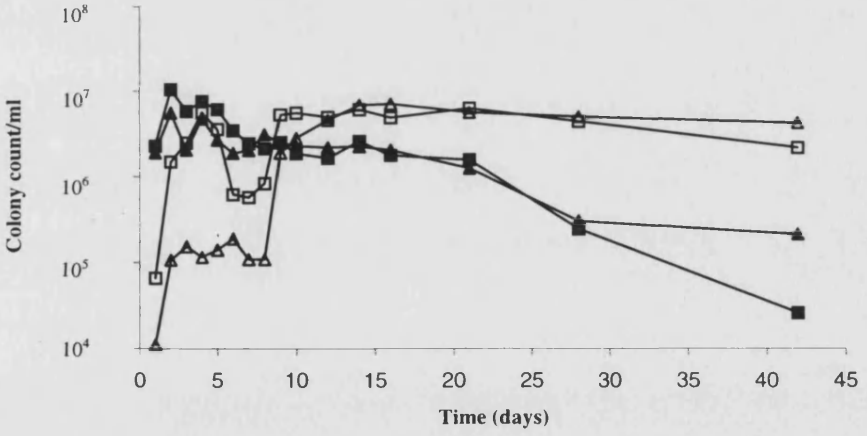


Figure 4.4.1. Growth curve (a) and cell viability (b) of *S. cerevisiae* strains AR27 (■,□) and *cwh30/kic1* (▲, Δ). Cultures were grown at 30°C in SC media with either 2% (w/v) glucose (■,Δ) or 2% (w/v) glycerol (□, Δ). Cells were transferred to solid YPD media and incubated for 48 h, 30°C. Resulting colonies were than counted. Mean from two separate experiments

lowest population size. Cell viability was higher in strains grown in glycerol than in strains grown in glucose (Fig. 4.4.1 (b)).

Intracellular trehalose levels were highest in *cwh30/kic1* grown in glucose than in the same strain grown in glycerol (Fig. 4.4.2). In contrast, trehalose levels increased in the wild type strain grown in glycerol (Fig. 4.4.2). Budding index (BI) was higher in *cwh30/kic1* strain when compared to AR27 grown in glucose (Fig. 4.4.3). Although BI was reduced in both strains when grown in glycerol, budding in the *cwh30/kic1* strain still remained slightly higher.

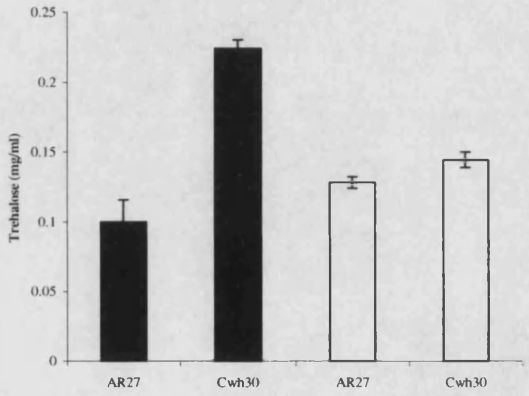


Figure 4.4.2. Intracellular trehalose levels (mg/mg cell dry weight) of strains grown in SC media containing either 2 % (w/v) glucose (■) or 2% (w/v) glycerol (□). Mean ± SEM.

When strains were grown in glycerol they have an increased resistance to Zymolyase (Fig. 4.4.3). Resistance or sensitivity to Zymolyase usually reflects changes in cell wall structure particularly in the β-glucan layer. Electron micrographs demonstrate that there is a change in cell wall composition in the cell walls of wild type strain, AR27, when grown with glycerol as the carbon source compared to glucose (Fig. 4.4.5). The cell walls of AR27 and *cwh30/kic1*

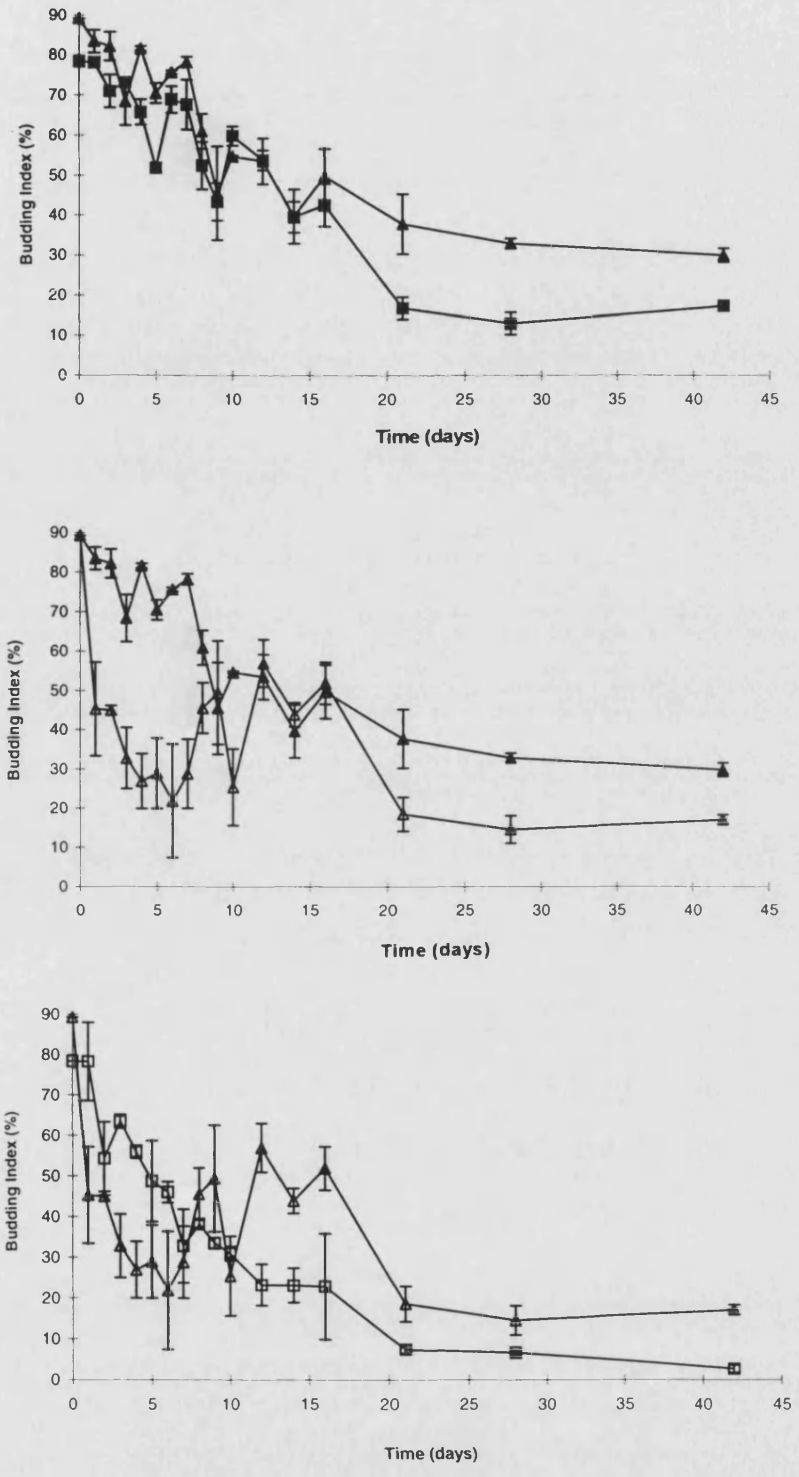
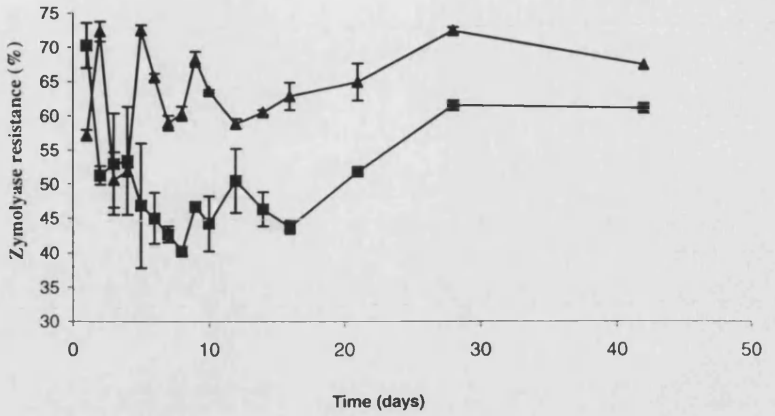
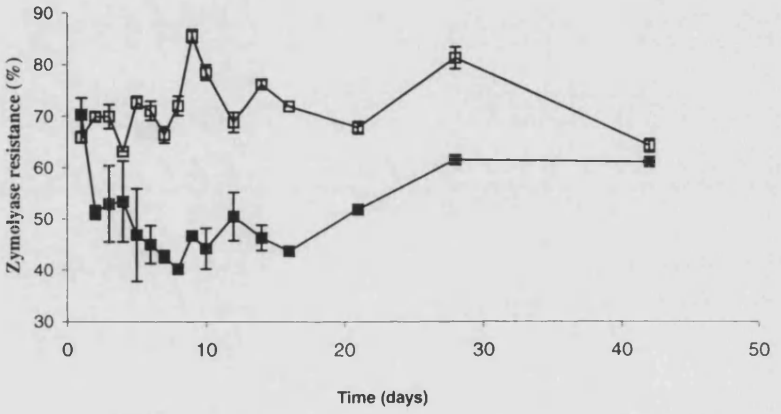


Figure 4.4.3. Budding index (%) of *S. cerevisiae* strains AR27 (■, □) and *cwh30/kic1* (▲, Δ). Cultures were grown at 30°C in SC media with either glucose (2% (w/v)) (■,▲) or glycerol (2% (w/v)) (□, Δ). Total number of cells were counted by haemocytometer. The number of unbudded cells was than subtracted from the total to allow the percentage of budded cells to be estimated. Mean ± SEM.





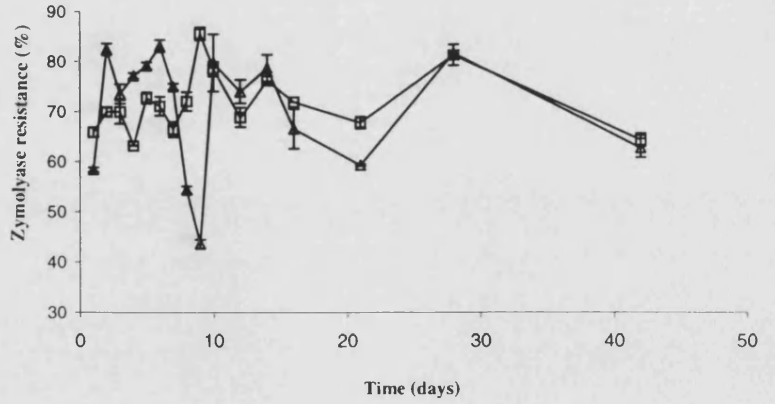


Figure 4.4.4. Zymolyase resistance (%) of *S. cerevisiae* strains AR27 (■, □) and *cwh30/kic1* (▲, Δ). Cultures were grown at 30°C in SC media with either glucose (2% (w/v)) (■, ▲) or glycerol (2% (w/v)) (□, Δ). Mean ± SEM.

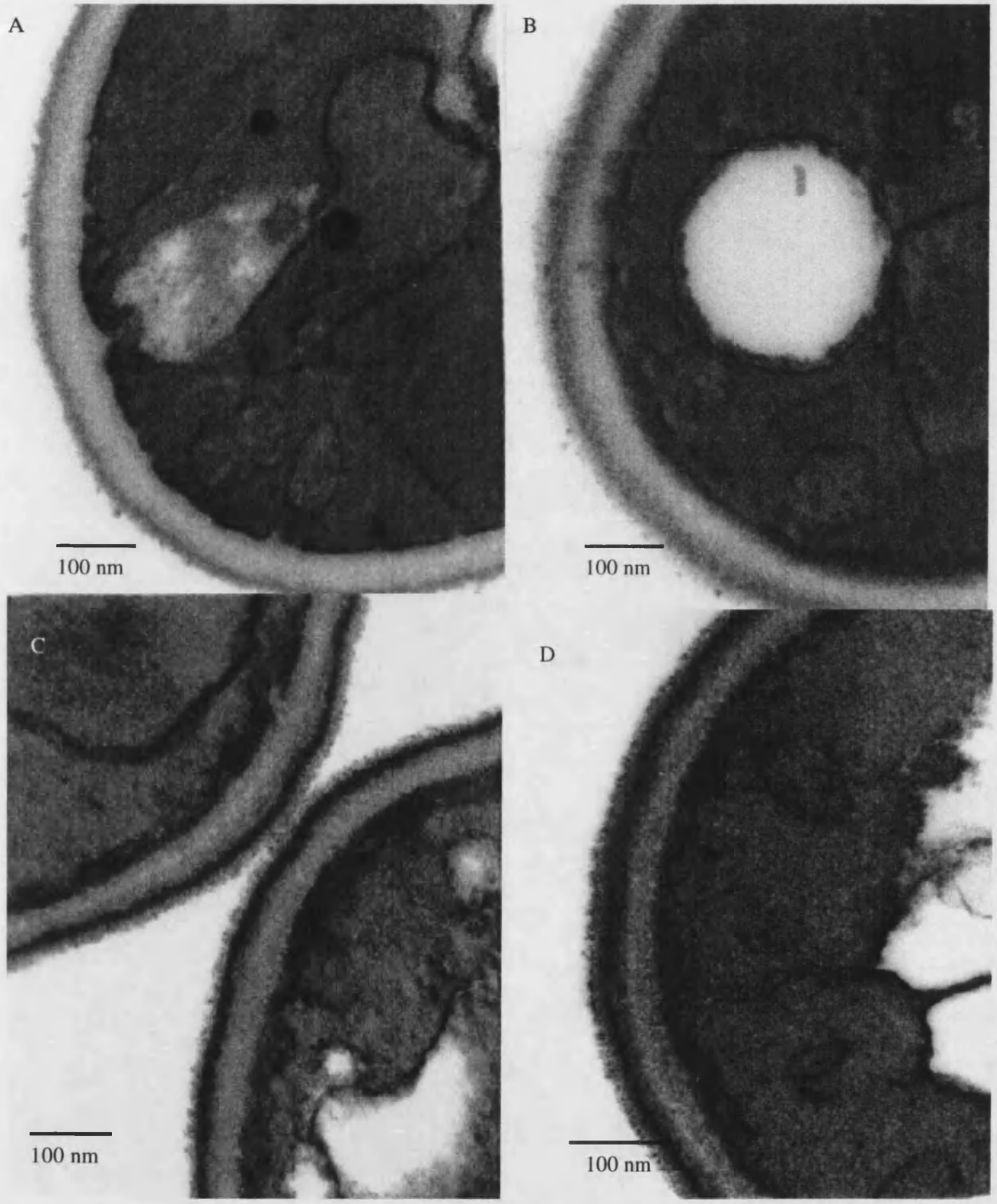


Figure 4.4.5. Transmission electron micrographs of cell walls in *S. cerevisiae* strains AR27 and *cwh30/kic1*. Cells were incubated (14 days, 28°C) in liquid YPD with either 2% (w/v) glucose (A (AR27) and B (*cwh30/kic1*)) or 2% (w/v) glycerol (C (AR27) and D (*cwh30/kic1*)) as a carbon source.

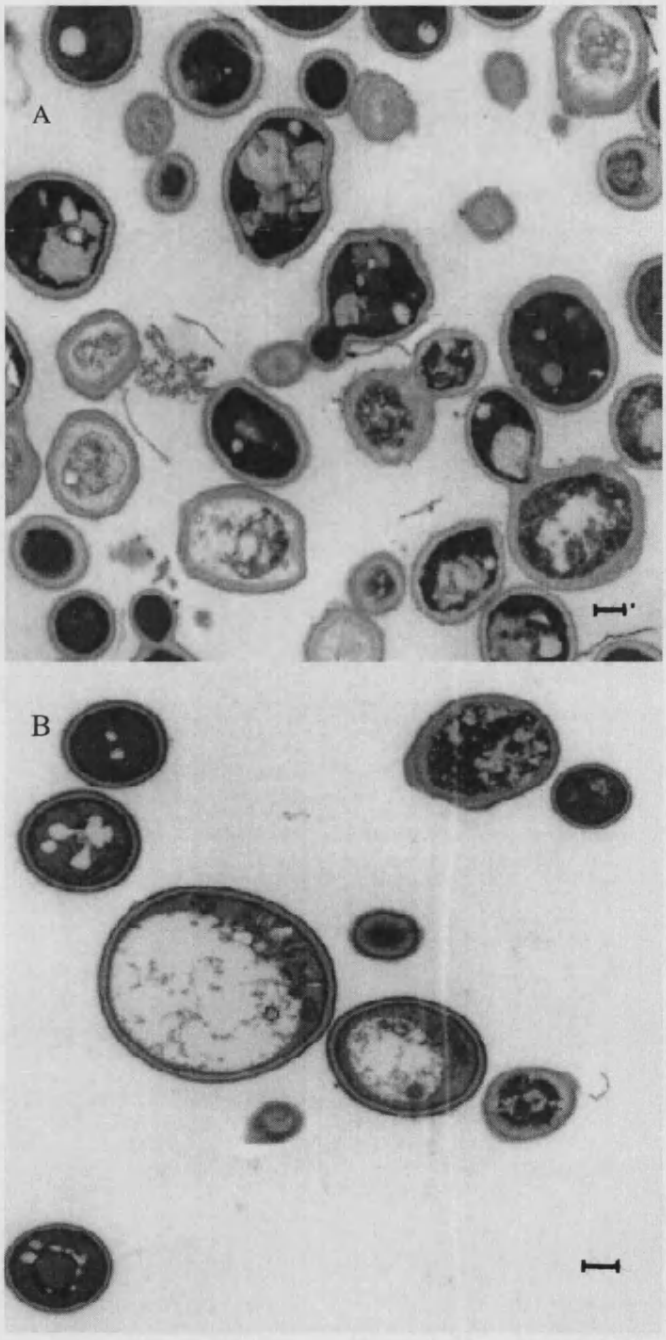


Figure 4.4.6. Transmission electron micrographs illustrating morphological differences between *S. cerevisiae* strain AR27 grown in (A) glucose and (B) glycerol as the carbon source. Cells were grown in liquid YPD containing 2% (w/v) glucose or 2% (w/v) glycerol for two weeks (28°C). Scale bar represents 1 μm.

take on a similar appearance under these metabolic conditions. The appearance of mannoproteins in the outer layer of the cell wall is denser in both AR27 and *cwh30/kic1* (Fig. 4.4.5). The morphology of cells grown in glycerol is also altered. They become larger with distinct nuclei (Fig. 4.4.6).

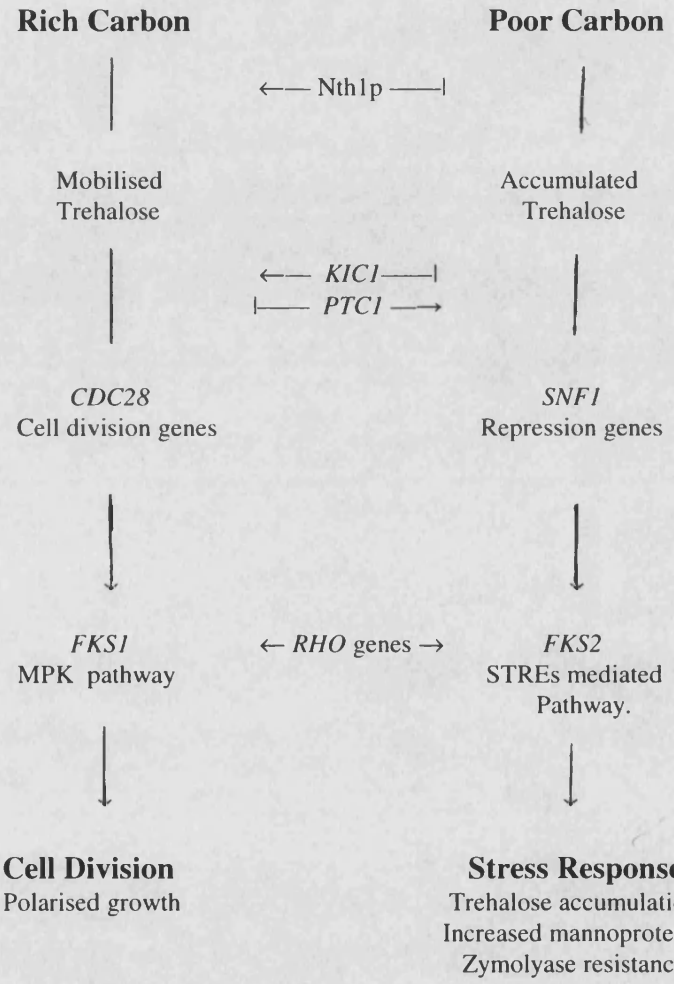
Similar findings to these have been reported on experiments with neutral trehalase mutants (Nwaka *et al.*, 1995). Neutral trehalase mutants also have an extended lag phase when transferred to media containing glycerol as a carbon source. Trehalose accumulation in yeast cells is usually associated with cells subjected to environmental stress or nutrient deprivation. In this instance trehalose accumulates in cells of a mutant that is defective in cell division and cell wall assembly. *cwh30* has a point mutation that affects *KIC1*, a calcium binding protein which is a yeast homologue of centrin. Yeast centrin is essential for the duplication of the spindle pole body (SPB) and strains with a mutation in this gene have an enlarged, unduplicated SPB. Although Kic1p interacts with Cdc31p it is not required for SPB duplication. Both Kic1p and Cdc31p are involved in cell integrity and morphogenesis. *KIC1* mutants are also thought to upregulate the HOG pathway. Recent studies with Calcofluor White resistant mutants have indicated that constitutive HOG functionality participates in the maintenance of cell wall architecture (Garcia-Rodriguez *et al.*, 2000). This study has revealed that trehalose accumulation may be associated with the HOG pathway.

This work also examines how a non-fermentable carbon source affects changes in cell wall structure and trehalose accumulation in *cwh30/kic1*. Here it has been

demonstrated that responses to stress follow a different pathway to nutrient deprivation. If the induction of stress characteristics in *cwh30/kic1* was attributed to an inability to ferment glucose then *cwh30/kic1* grown in glycerol would have a similar phenotype to when it is grown in glucose. The extended lag phase, increased population size and reduced intracellular trehalose content this strain displays when it is grown in glycerol implies that it is following a pathway that is metabolically independent from fermenting cells grown in glucose. The phenotypes *cwh30/kic1* adopted in fementative and nonfermentative growth could be resulting from a reduced ability to mobilise trehalose.

Cells subjected to stress usually have increased resistance to Zymolyase. Here, the high resistance shown by *cwh30/kic1* could be due to upregulation of the HOG pathway. During exponential growth *cwh30/kic1* has significantly increased sensitivity to Zymolyase. This reveals that cell wall construction is adversely affected in this strain. Electron micrographs reveal a disorganisation of the cell wall that leads to defective budding and bud separation (Sullivan *et al.*, 1997). It has already been found that *KIC1* mutants lyse at the bud neck (Sullivan *et al.*, 1998). In this study, developmental defects in bud emergence have been found in this strain which are probably caused by a reduced β-glucan layer and disorganisation of actin localisation. This mutant has a similar phenotype to *NTH1* mutants which also show reduced growth on glycerol (Nwaka *et al.*, 1995). Neutral trehalase activity may have a direct correlation with cell wall construction and could be one of the regulators in a cell integrity response towards stress.

Figure 4.4.7. The mobilisation of trehalose in a rich carbon source. Nth1p activity increases and leads to the mobilisation of trehalose. In a poor carbon substrate, Nth1p activity is reduced and *FKS2* expression replaces *FKS1*.



4.5 Trehalose accumulation in *fks1* and *gas1*, strains defective in cell wall assembly and cell separation.

To further investigate the nature of trehalose accumulation in cell wall mutants the levels of trehalose accumulation were also measured in two other mutants defective in cell wall construction *fks1* and *gas1* (Fig. 4.5.1.). Both of these strains are Calcofluor White hypersensitive and are known to lose viability in stationary phase but neither are resistant to *K1* killer toxin (Ram *et al.*, 1995). There was an increased level of intracellular trehalose in *fks1* when compared to the parental strain AR27 but *gas1* had a reduced level of trehalose (Fig. 4.5.1).

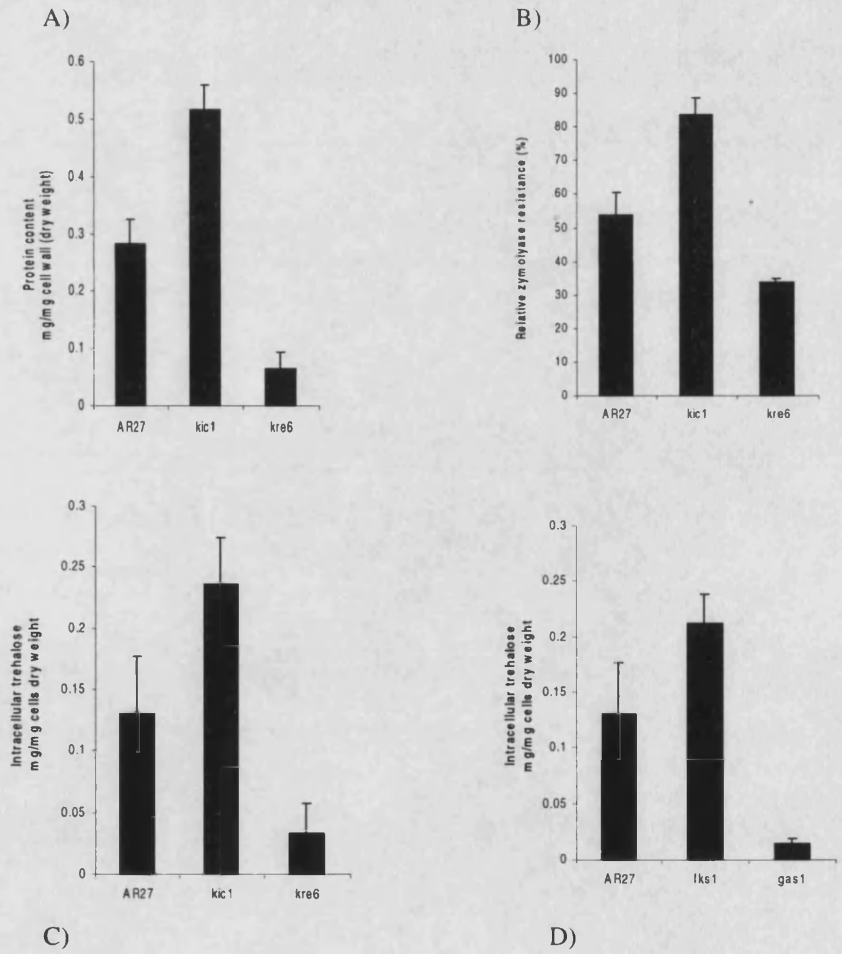


Figure 4.5.1. Cell wall protein content (A) and zymolyase resistance (B) in AR27 (parental strain) and cell wall mutants *kic1*, *kre6*. Intracellular trehalose content of AR27 (parental strain) and cell wall mutants C) *kic1*, *kre6* and D) *fks1*, *gas1*.

FKS1 encodes a protein that is a component of the β1,3-glucan synthase (GS) complex, involved in the synthesis of the β1,3-glucan structural component of the cell wall (Ram *et al.*, 1995; Zhao *et al.*, 1998). Mutants which contain an *fks1* mutation have a 75% reduction in cell wall β1,3-glucan (Ram *et al.*, 1995). *FKS1* is expressed under optimal growth conditions whereas an alternative component, *FKS2*, is expressed under certain stress conditions, in response to mating pheromone, during growth in poor carbon sources and in *fks1* mutants. On entering stationary phase, as a consequence of glucose depletion, *FKS2* is strongly expressed and is under the control of the *SNF1* regulated *MIG1* transcriptional repressor (Zhao *et al.*, 1998). Induction of *FKS2* is usually accompanied by an equivalent decrease in *FKS1* but this does not appear to affect GS activity within the cell (Zhao *et al.*, 1998). This has led to the theory that there may be two different GS complexes; one predominates in exponential growth and the other is induced in response to stress (Zhao *et al.*, 1998). If Fks1p has a direct role as a catalytic subunit of the glucan synthase complex, it is not involved in the direct binding of glucose as it does not contain a UDP-glucose binding site (Ram *et al.*, 1995). In addition the *in vitro* activity of GS in *fks1* mutants is comparable to wild types (Ram *et al.*, 1995). The amino acid sequence of *FKS1* predicts an integral protein that has a multiple transmembrane structure therefore Ram *et al.* (1995) suggest that it could function as a channel-like structure in the membrane in a similar way to glucose transporters.

GAS1 encodes a gene for a GPI-anchored membrane glycoprotein believed to be involved in morphogenesis (Ram *et al.*, 1995; Popolo *et al.*, 1997). This mutation is lethal when crossed with *KRE6* (Popolo *et al.*, 1997). Cells with a *GAS1*

disruption release proteins in to the media and have increased cell wall levels of chitin and mannan (Ram *et al.*, 1998). Additionally they also show increased GS activity (*Ram et al.*, 1998). It is thought that Gas1p may be involved in the retention of β1,3-glucan as media have been found to contain five times more β1,3-glucan than in wild type cells (Ram *et al.*, 1998).

This study reveals that mutations in *FKS1* and *GAS1* create an imbalance in the intracellular ratio of carbohydrates. *FKS1* has a high intracellular ratio of trehalose brought about by the loss of intracellular glucose. By contrast *GAS1* has a low intracellular trehalose level with a high intracellular glucose level. These findings confirm that both *FKS1* and *GAS1* are proteins intrinsically linked with the incorporation of glucose into the cell wall. Defects in *FKS1* reduce the availability of glucose for cell wall construction possibly by breaking the pathway that allows the successful transportation of glucose to the cell wall. This would lead to *FKS2* induction under the control of the *SNF1* regulated *MIG1* transcriptional repressor. The cell wall would therefore take on a similar conformation to that found in non-dividing cells. This mode of cell wall construction probably allows the attachment of a greater number of GPI anchored CWP's for stress protection but prevents the efficient deposition of chitin within the cell wall that is required for successful bud separation and cell division. Many strains harbouring mutations that lead to an inappropriate switch from the cell integrity pathway to a stress response (e.g., *cwh30/kic1*), appear to have a similar phenotype of abnormal chitin deposition, cell lysis and abnormal bud separation. Repression of *PKC1* leads to extensive cell lysis, accompanied by the release of 45% of cellular protein into the medium (Zhang *et al.*, 1999).

Trehalose appears to accumulate in strains where a mutation involves a cell wall function associated with a specific cell integrity pathway. In several other strains with genes involved in cell integrity and morphogenesis such as *gas1* and the *K1* killer resistant strain, *kre6,* there is a decrease intracellular trehalose level when compared to wild type strains under optimal growth conditions (Fig.5.4.1). *KIC1* is a vital gene required for exponential growth in optimal conditions. A reduction in levels of Kic1p results in cells exiting from the normal developmental pathway and adopting a stress response. These results demonstrate that these stress characteristics may be a contributing factor to the cell's ability to survive long term starvation. The *kre6* strain does not adopt resistance to cell wall degrading enzymes or increased CWP content and therefore is not as successful at withstanding long term starvation as AR27 or *kic1* (Fig.4.5.3).

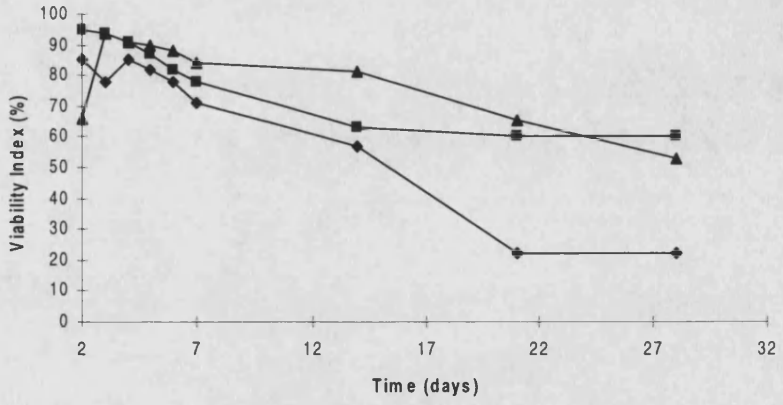


Figure 4.5.3. Cell viability of *S.cerevisiae* strains AR27 (■), *kic1* (▲) and *kre6* (♦). Cultures were grown at 30°C in SC media with 2% (w/v) glucose. Viability was assessed by colony counts subtracted from total cell number. Mean from two separate experiments.

Trehalose serves as a molecular protectant by preventing proteins from denaturing in extreme physical conditions (Singer and Lindquist, 1998). However

its continued presence interferes with protein refolding. This would explain the need for its mobilisation prior to exponential growth. This work illustrates the opposing relationship between genes involved in exponential growth and those involved in the stress response that lead to trehalose accumulation in *S. cerevisiae*.

4.6 Chitin levels in the cell wall of *kre* strains.

Although chitin is only present in small quantities within the cell wall, it is essential for cell growth (Shaw *et al.*, 1991). During vegetative growth it is first deposited as a ring at the incipient bud site and the base of the emerging bud. Following nuclear separation and cytokinesis a thin disk is deposited within the ring to form the primary septum. This is then reinforced with a secondary septum composed of mannan and glucan. Cell separation occurs when the primary septum digested by chitinase leaving a budding scar, composed of the initial chitin ring, on the mother cell (Fig.4.6.1.).

Chitin content of the *kre* strains varies considerably. The *cwh47/ptc1* strain, which has aggregated cells with a separation defect and does not exit G1, contained the least amount of chitin. The other *kre* strains gave higher chitin levels then the parental strain AR27. The mutation in the *cwh30/kic1* strain causes delocalised actin and a cell separation defect. Chitin deposition in the primary septum is clearly visible in electron micrographs (Fig.4.6.1). These results support the theory that chitin can partly compensate for a reduction in β1,6-glucan and allow GPI-cell wall proteins to attach to the cell wall. The high

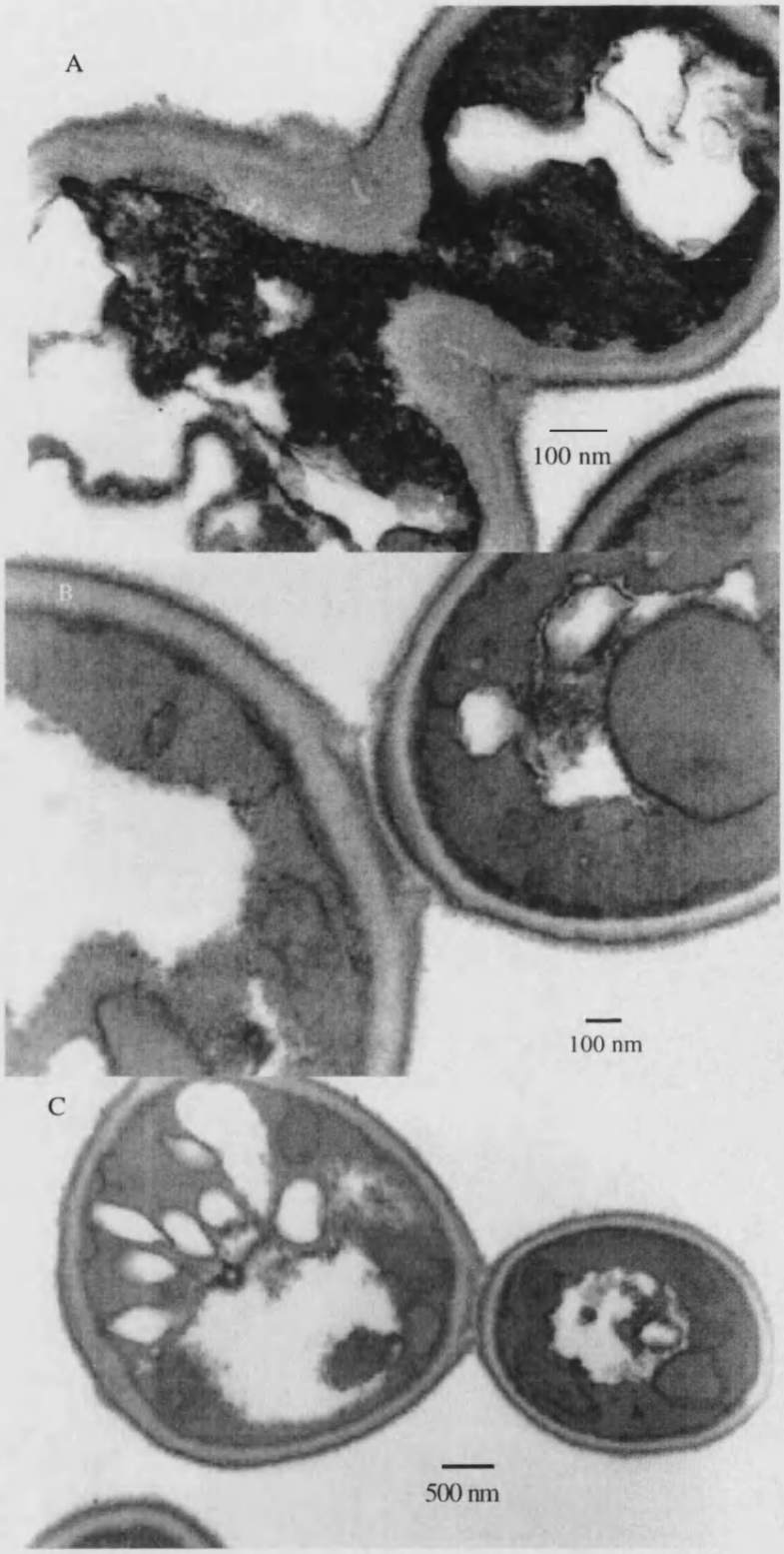
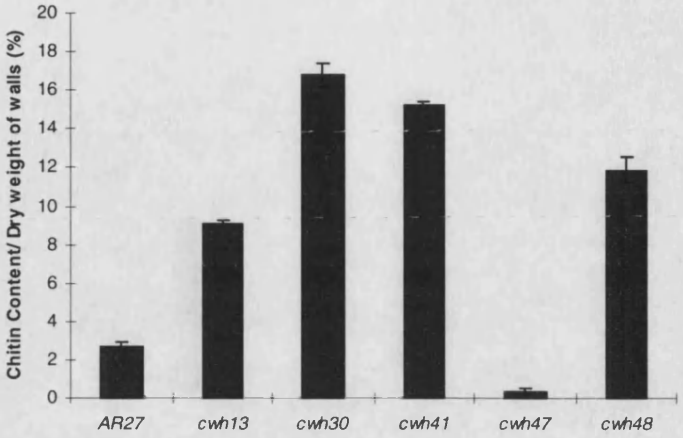


Figure 4.6.1. Transmission electron micrographs of *S. cerevisiae* in the process of cell division (A) and separation (B and C). Chitin encircles the junction between mature cell and the newly created bud to allow cytoplasm to be extruded into the newly formed cell.

a)



b)

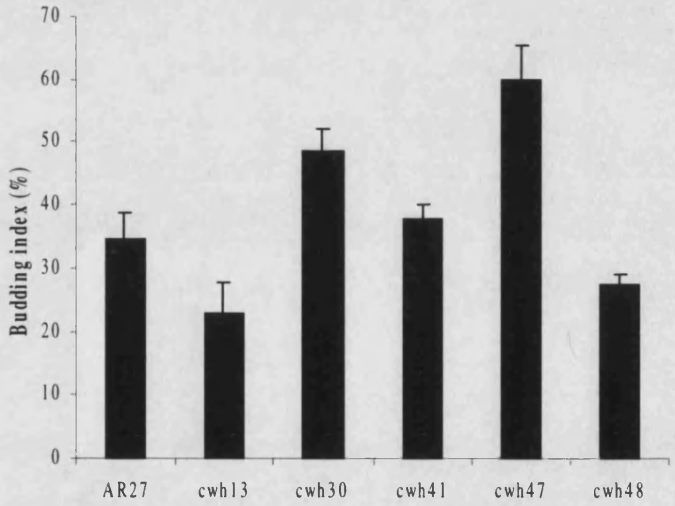


Figure 4.6.2. Chitin content (a) and budding index (b) of *kre* strains after 2 weeks incubation. The chitin content of *kre* strains was quantified by acid hydrolysis. Strains were incubated overnight at 100°C in 6M HCl and then assayed colorimetrically for glucosamine content. Mean ± SEM.

levels of chitin discovered in *cwh30/kic1* and *cwh41/gls1* offer an explanation as to why there is not a reduction of cell wall proteins in these strains despite a reduction of β1,6-glucan. Increased level of chitin were also found in *cwh13/erd1* and *cwh48/kre6* although levels of cell wall proteins were found to be lower than in the other *kre* strains (Popollo *et al.*, 1997). This could be due to a defect in the localisation of the glycoproteins rather than by the way they are attached to the cell wall.

Several cytoskeletal elements are believed to play a role in chitin deposition and regulation. Spatial control of chitin to the budding ring is thought to involve 10-nm septin filaments lying underneath the plasma membrane at the bud emergence site (Roberts *et al.*, 1983). If a mutation occurs in any of the genes responsible for these proteins (*CDC3, CDC10, CDC11* and *CDC12*) chitin is deposited in a diffuse ring (Roberts *et al.*, 1983). Proteins that are involved in cell polarity and the cytoskeleton also influence the deposition of chitin in the cell wall. Mutations affecting actin function, have irregular deposition where chitin is diffused over the cell surface and not restricted to the cell neck (Rodriguez and Paterson, 1990). Additionally there is a correlation between actin delocalisation and chitin deposition in several bud emergence and polarity mutants, *cdc24, cdc42, bem2, rho3* and *rho4* (Sloat *et al.*, 1981; Adams *et al.*, 1990; Matsui and Toh-e, 1992).

4.7 The transformation frequency of *kre* strains in relation to phenotype.

All the *kre* yeast strains were transformed with *kanMX4* DNA fragment, pUG36, YPB1-ADHpt and pRS426-HA::SED1. Transformation frequency of *kre* strains varied significantly (Fig. 4.7.1). *AR27* was successfully transformed with all of

the plasmids. Transformants were only obtained from *cwh30/kic1* after incubation at 42°C was increased to 45 min. Transformation frequency was highest in *AR27*, *cwh41* and *cwh13* and lowest in *cwh47* and *cwh48*. The transformation frequency of *cwh*47 and *cwh*48 may improve if cells are grown with sorbitol. All of the strains were transformed with YPB1-ADHpt. The yEGFP gene is controlled by an alcohol dehydrogenase (*ADH1*) promotor which is normally induced by fermentative growth on glucose (Ruohonen *et al*, 1995). The yEGFP (yeast-enhanced green fluorescent protein) could be seen visually by fluorescence microscopy in all of the strains except for *cwh30/kic1* (Figure 4.7.2).

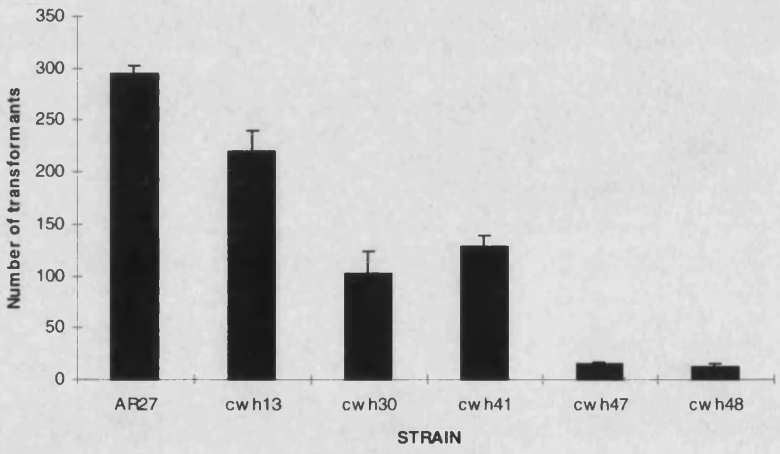


Figure 4.7.1. Transformation frequency of *kre* strains. Strains were transformed with the plasmid YPB1-ADHpt using LiAc to create competent yeast cells (Mean ± SEM).

Interestingly, fluorescence microscopy revealed that only a proportion of cells are in a growth phase which allowed yEGFP to be expressed. The majority of these cells appear to be in the process of budding and cell division. Alternatively, lack of expression could be because a proportion of the cells have aborted the plasmid.

Although the *cwh30/kic1* strain was successfully transformed with the yEGFP gene, the *ADH1* promoter is most probably not induced as GFP cannot be

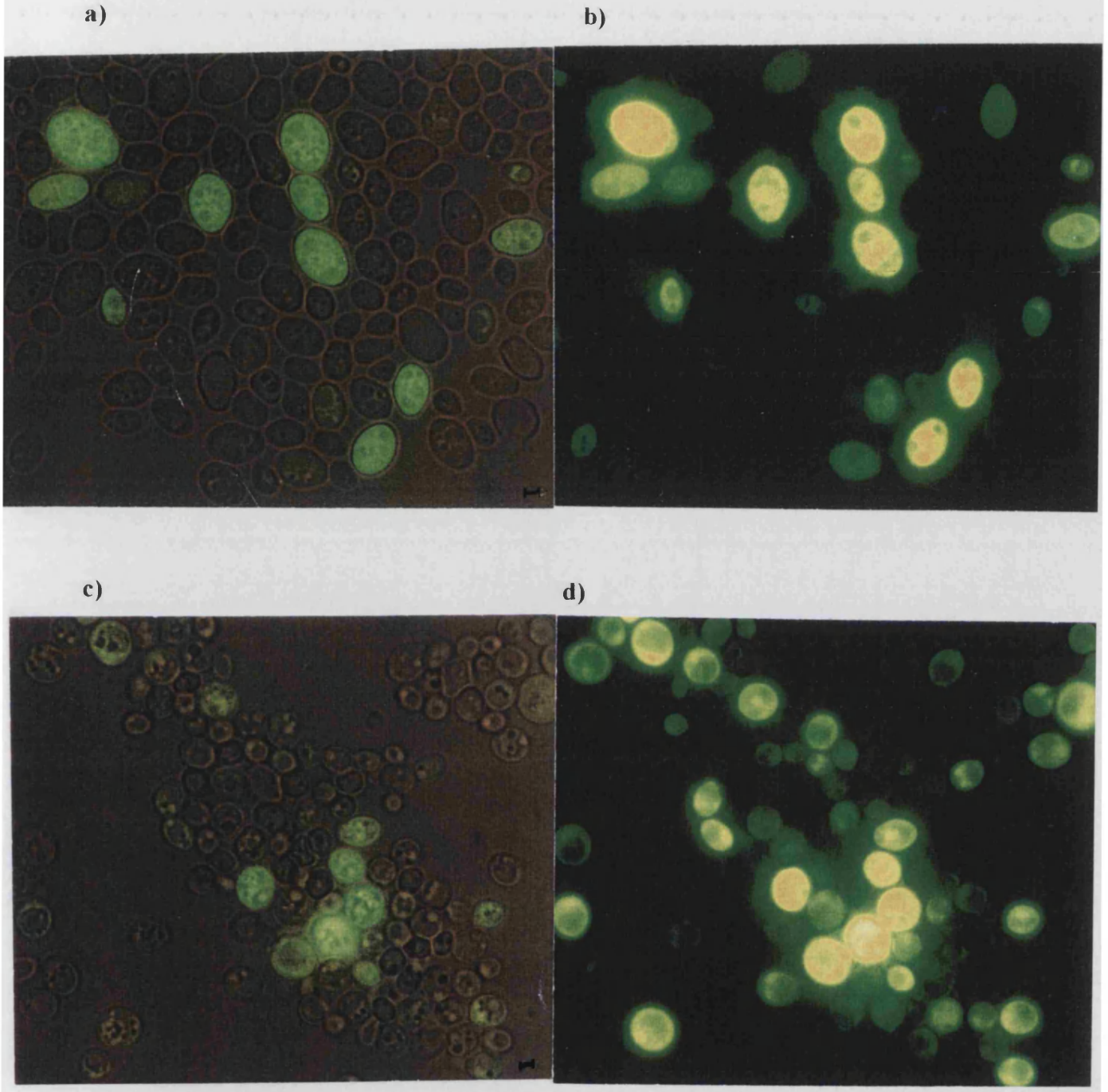
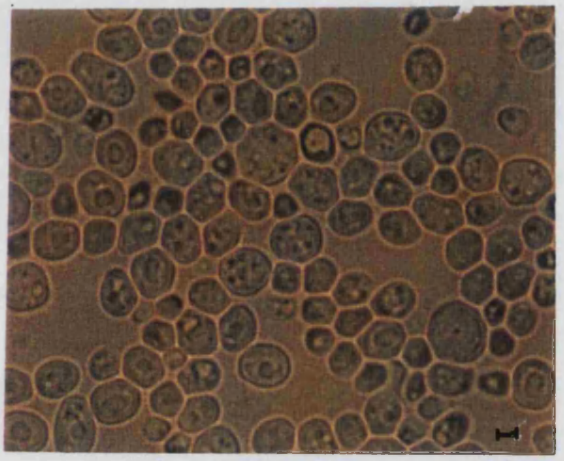


Figure 4.7.1. Fluorescence micrographs of cells transformed with YPB1-ADFpt. The yEGFP gene is controlled by the *ADH1* promoter. Cells were incubated on solid medium (SC-URA) then transferred to glass slides without fixation. Cells were then photographed on a fluorescent microscope at a wavelength of 509 nm with and without background illumination.
Scale bar represents 1 μm.
a, b: AR27 **c,d:** *cwh13/erd1* **e:** *cwh30/kic1p,* **f,g:** cwh41/gls1 **h,I:** *cwh47/ptc1* **j:** *cwh48/kre6.*

e)



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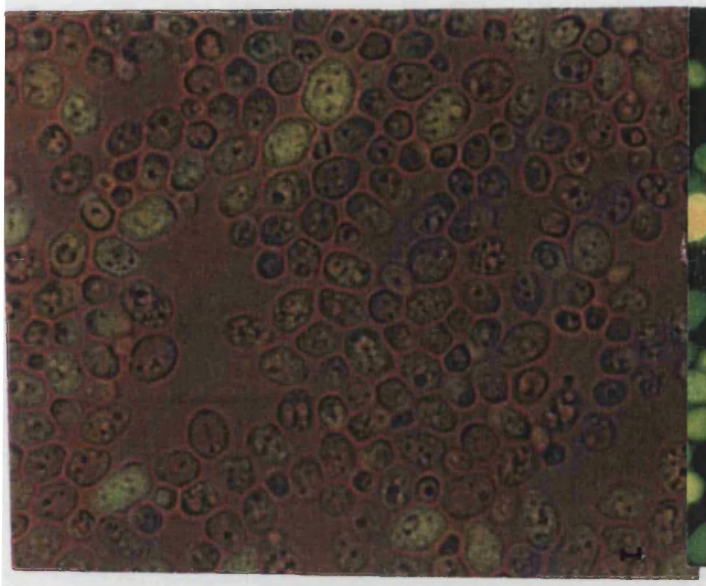

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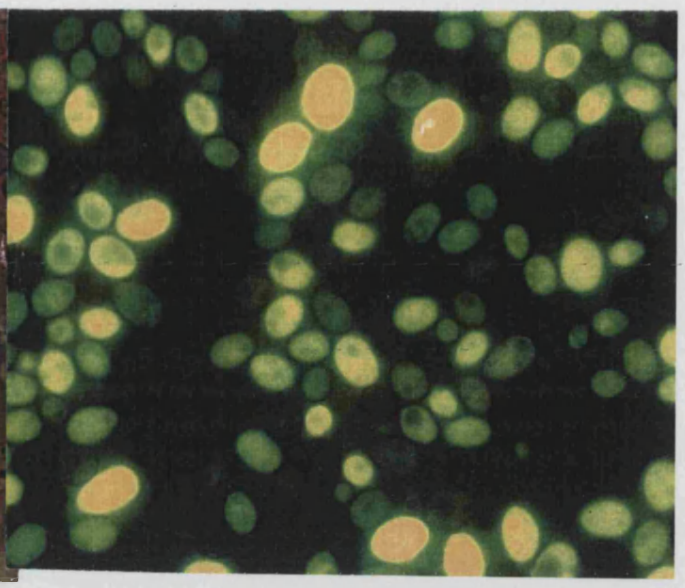

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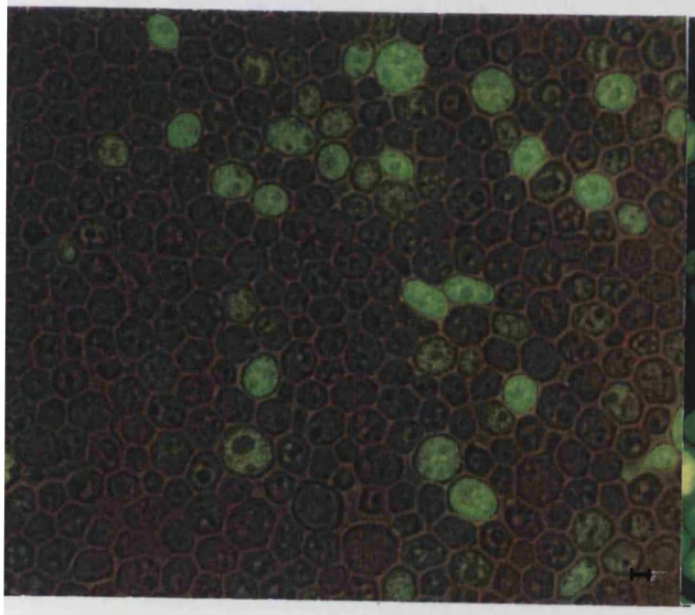

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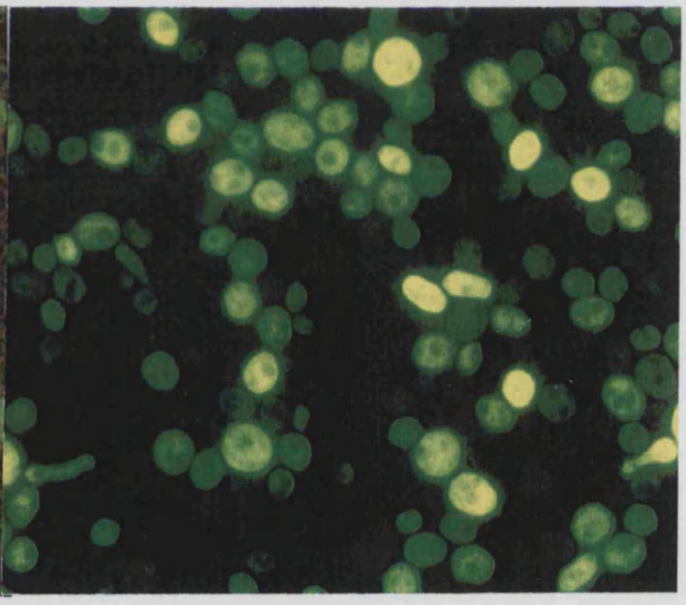

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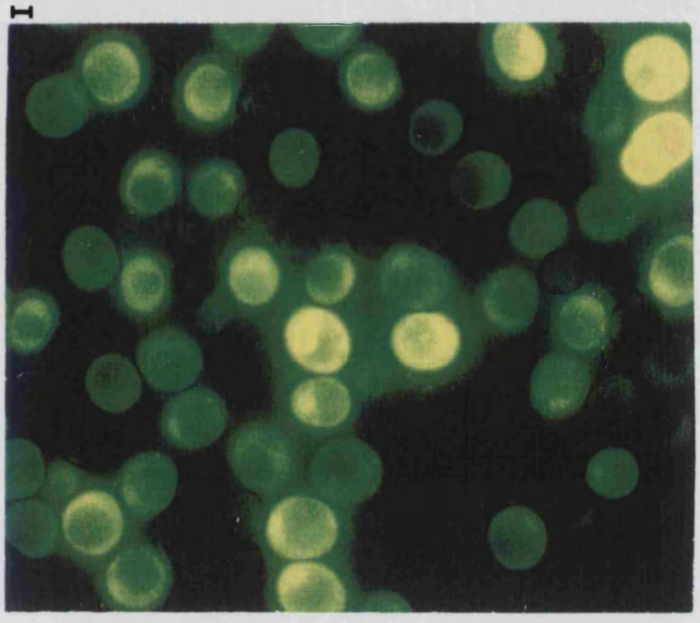


Figure 4.7.1 (continued)

detected in this strain. Therefore a mutation in *KIC1* indirectly affects induction of *ADH1* and the ability for cells to grow in a fermentative carbon source. This supports the theory that *cwh30/kic1* is following a different cell integrity pathway to the other *kre* strains.

4.8 Mechanisms of copper ion uptake in *S.cerevisiae* and its relationship with Cu^{2+} resistance in *cwh48/kre6.*

In this study one strain, *cwh48/kre6*, showed an elevated resistance to copper toxicity (Fig 4.8.1). However, this strain did not also appear to be resistant to oxidative stress as it was not sensitive to diamide. *cwh48* has enlarged cells which is probably attributed to the weakened cell wall glucan matrix. The internal volume of the cell would therefore be increased which would perhaps lead to a shortage of essential metabolites required for respiration and metabolism. Increasing the levels of Cu^{2+} may compensate for a reduced concentration of these ions in this strain. The weakened cell wall may also contribute to increased permeability and a greater loss of essential metabolites into the medium.

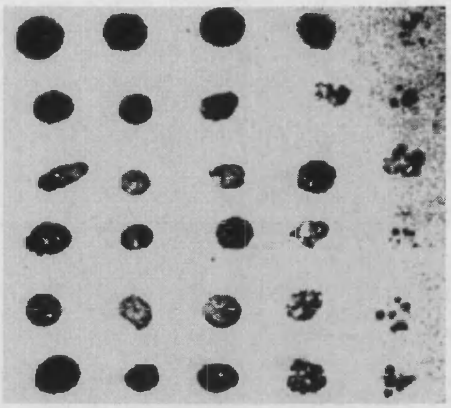
All the *kre* strains were found to be more sensitive to Calcofluor White than the parental strain AR27, although the degree of sensitivity varied between each strain. *Cwh13-1/erd1* had the greatest sensitivity. None of the strains were sensitive to diamide. Slow growth on diamide would have suggested that the strains were sensitive to oxidative stress. On $CuSO_4$ selection plates, *cwh*48 had a resistance higher than the parental strain AR27 (Fig. 4.8.1). C*wh48* also had

the lowest cell viability but budding index was similar to AR27 (Fig. 4.8.2). Cell size was significantly larger in *cwh48* than in AR27 (Fig. 4.8.2, Fig. 4.8.3).

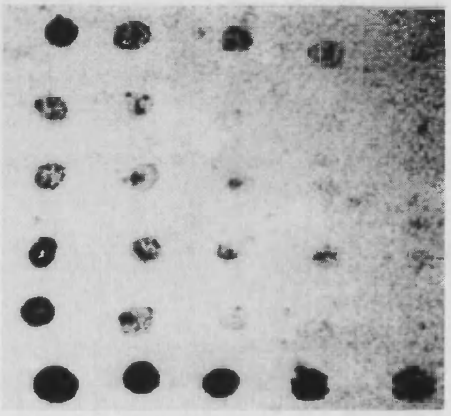
Copper ion uptake in *S. cerevisiae* involves *CTR1*. Mutations in *CTR1* lead to defects in high affinity copper transport and copper deficient phenotypes. These include poor growth in copper deficient medium, reduced levels of Cu,Zn superoxide dismutase (SOD) and defects in respiration and high affinity Fe transport. Ctr1p is a plasma membrane glycoprotein which has several repeats of a methionine-containing copper-binding domain within the amino terminus. On entering yeast cells, copper is distributed to copper requiring proteins, such as Cu,Zn SOD, cytochrome oxidase and Fet3p.

Protection against elevated levels of copper in *S. cerevisiae* is offered by two genes *CUP1* and *CRS5* which encode isoforms of a metal binding protein called metallothionein (MT). Yeast MTs are small proteins, highly enriched in cysteine residues, similar to those found in other higher eukaryotes. Each MT polypeptide binds 6-7 copper atoms. Copper sequestration by MT makes it unavailable for reactive oxygen species (ROS) redox chemistry. Elevated levels of copper are first recognised by the gene product of *ACE1*, a Cu^{2+} metalloregulatory transcription factor. Ace1p is a 225 amino acid protein with a highly basic amino terminal region (110 amino acids) which encompass a copper-activated DNA binding domain. The *ACE1* DNA binding domain undergoes a copper dependent conformational change and can recognise small fluctuations in copper concentration. Ace1p not only activates MT transcription but also activates *SOD1*.

A) *S.cerevisiae* strains grown on SC solid media.



B) *S. cerevisiae* strains grown on SC solid media with 1.5 mM $CuSO_4$.



C) *S. cerevisiae* strains grown on SC solid media with 3.0 mM $CuSO_4$.

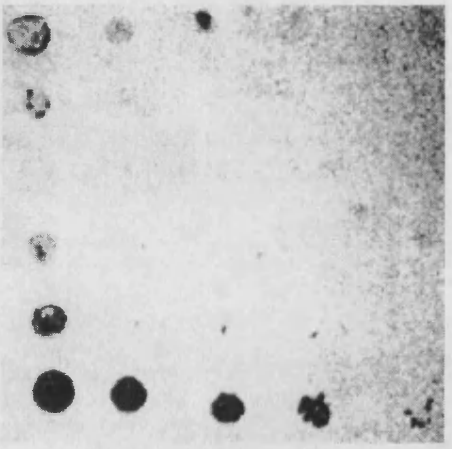


Figure 4.8.1. Copper sulphate resistance in *cwh48/kre6*. Strains were first grown in liquid YPD (14 days, 28°C), then transferred to SC media containing the following quantities of copper sulphate, 0 (A), 1.5 mM (B) or 3.0 mM (C) and incubated for 48h (28°C).

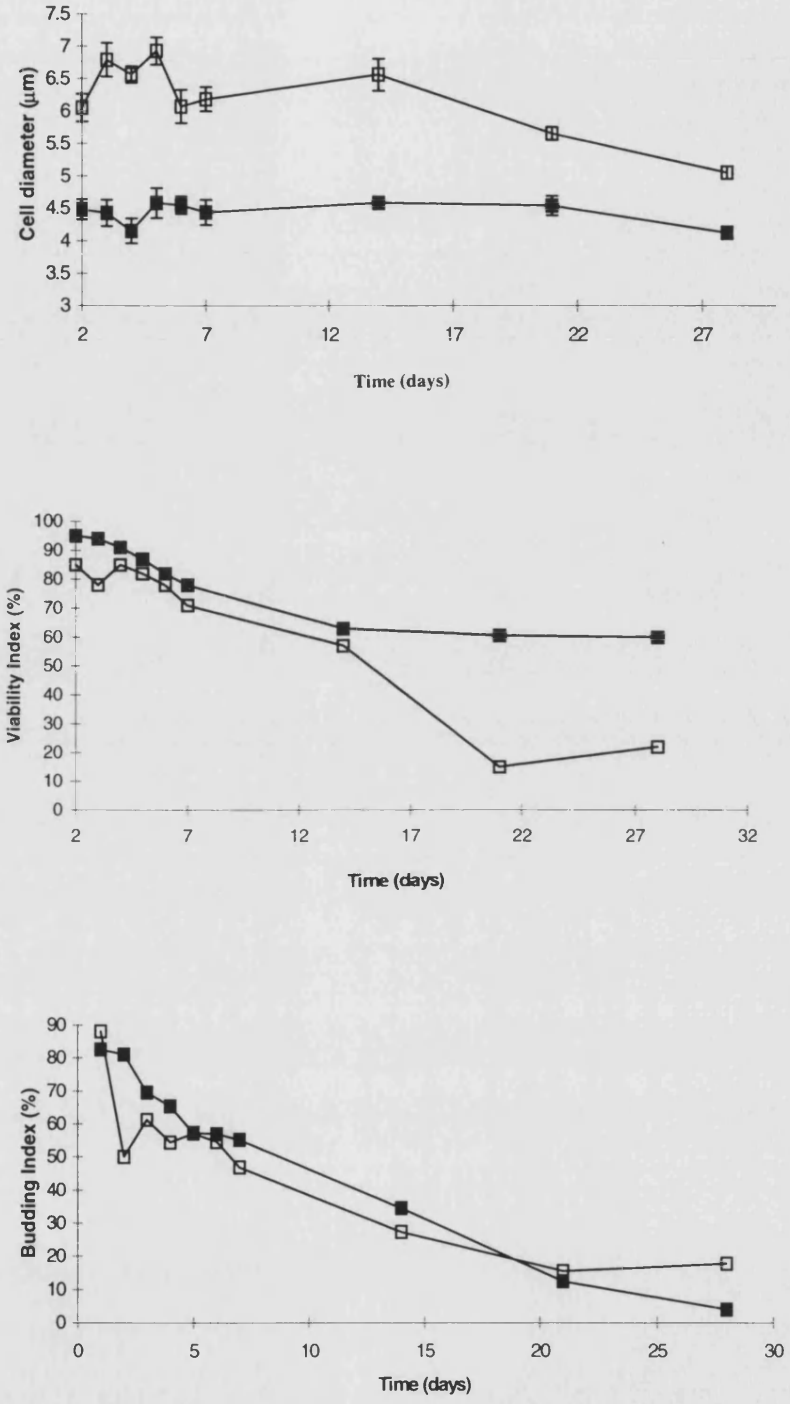


Figure 4.8.2 Growth parameters of AR27 (■) and *cwh48/kre6* (□). Strains were grown in SC liquid media (2% (w/v) glucose). Cell diameter (mean ± SEM) was measured by image splitting microscopy.

Figure 4.8.3. Viability assessment of *cwh48/kre6.* Cells were stained with methylene blue (0.01%) and sodium citrate (2%). Viable cells remain unstained. Scale bar represents 1 μm.

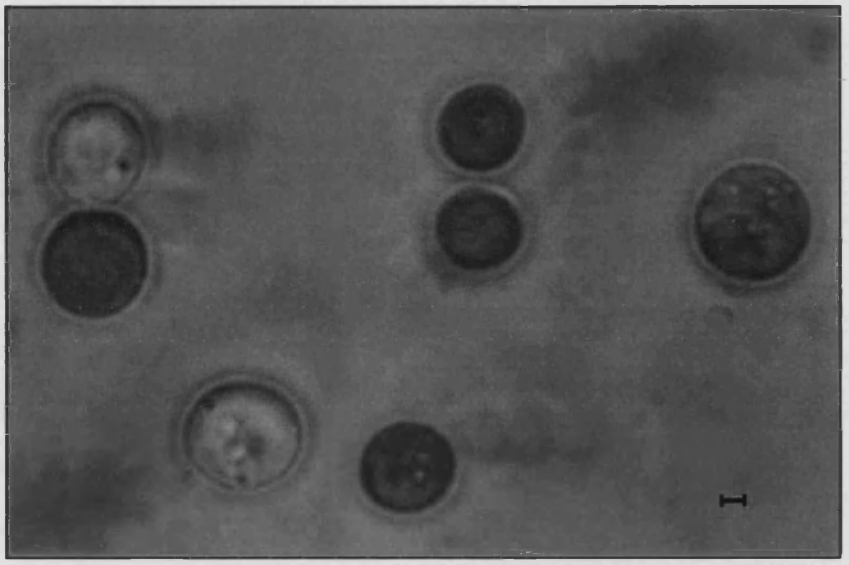
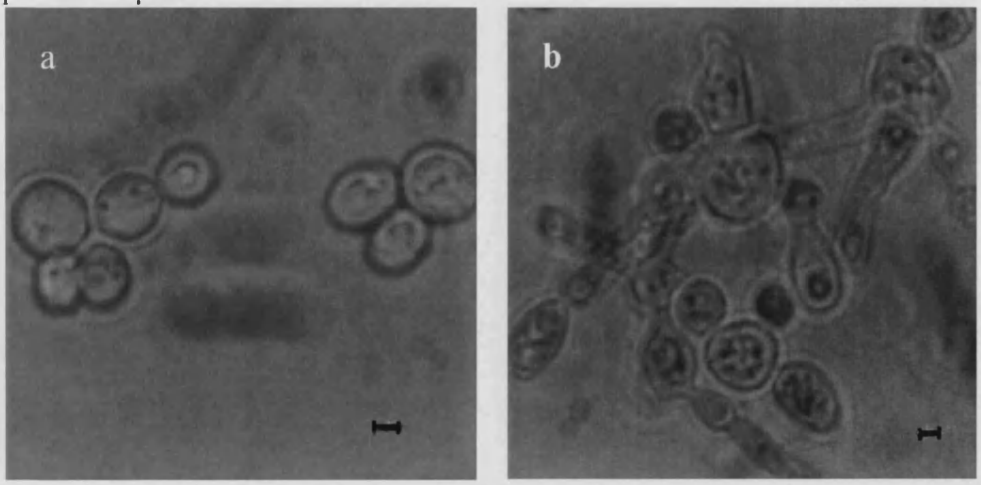


Figure 4.9.1. Viability assessment of *cwh47/ptc1.* Cells fail to produce stationary phase characteristics and have severe morphological defects. Methylene blue stained cells from cultures grown for 1 week (**a**) and 5 weeks (**b**). Scale bar represents 1 μm.



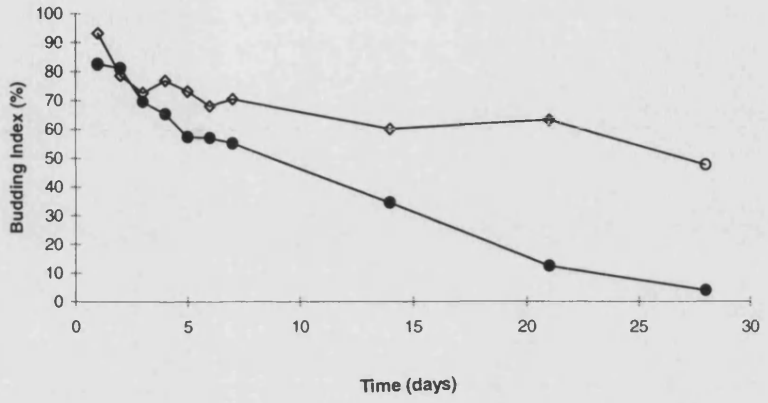
Improved growth in *cwh48/kre6* due to elevated copper levels in the media could be due to increased copper transportation into the cell. The transportation of copper into the cell is by an active process that involves CWPs. These processes could be restricted by cell wall defects that influence the level of CWPs.

4.9 Lack of cell integrity and morphogenesis in *cwh47/ptc1* prevent cells from entering stationary phase.

The *cwh47/ptc1* mutation affects cell integrity. Cells have an irregular morphology and do not separate from the mother cell after bud formation (Fig. 4.9.1). After 3 weeks the population had a low number of spherical single cells and therefore the budding index was high (Fig 4.9.2). The number of methylene blue stained cells also increased at 3 weeks. This indicates reduced viability (Fig.4.9.1). This growth defect did not seem to decrease resistance to Zymolyase. *PTC1* deletants exhibit multiple phenotypes: temperature-sensitive growth, accumulation of unspliced precursor tRNAs, sporulation defects, and failure of cell separation during mitotic growth.

Zymolyase resistance was generally greater in *kre* strains that had been grown with 1M sorbitol added to the medium. This indicates that high osmolarity induces changes in cell wall construction. In two of the strains *cwh30/kic1* and *cwh47/ptc1* there is very little difference in Zymolyase resistance. This could indicate that cell wall construction is not being regulated and that cell integrity in response to the surrounding environment has not been initiated. In this study, both *cwh30/kic1* and *cwh47/ptc1* have an increased budding index and morphological defects related to media osmolarity. These strains both have a reduced ability to regulate the HOG pathway.

a)



b)

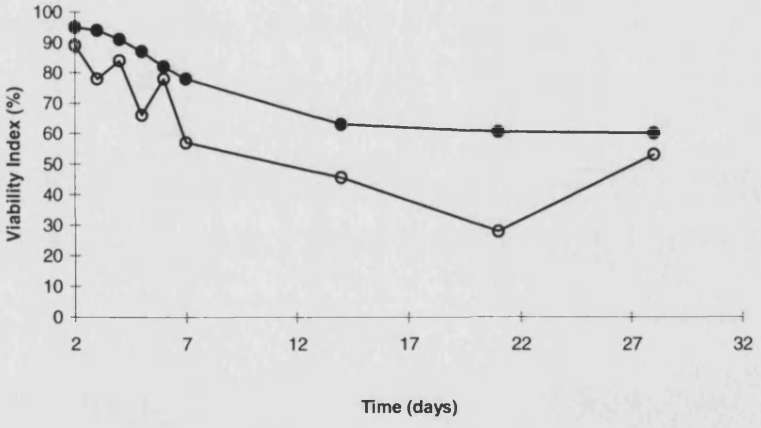


Figure 4.9.2. Growth parameters a) budding index and c) cell viability in AR27 (●) and *cwh47/ptc1* (O). Strains were grown in SC liquid media.

4.10 Summary.

Analysis of *kre* strains reveal they have mutations which influence levels of β1,6-glucan found in the cell wall. Functional analysis reveals that these mutations occur either in the secretory pathway (e.g., *erd1, cwh41*) or are involved in cell wall construction and regulation (e.g., *ptc1, kic1, kre6*). Transformation frequency, Zymolyase resistance and trehalose accumulation vary in the *kre* strains studied. This can be attributed to the influence that a particular mutation has on the cell wall and cell metabolism. Zymolyase sensitivity is also shown in strains which are already known to lose viability in stationary phase (eg., *bcy1-1, ard1* and *ubi4).* In these strains and in one of the kre strains (*cwh13/erd1*) resistance to Zymolyase increases as stationary phase progresses. This is probably due to changes in the physiological characteristics brought about by the induction of different cell integrity pathways.

Two of the *kre* strains, *cwh30/kic1* and *cwh47/ptc1*, manifested very different characteristics due to an inability to respond to changing environment. *cwh30/kic1* displayed stress characteristics despite growing in optimal growth conditions, while in low osmolarity, *cwh47/ptc1* failed to adopt the characteristics that would confer resistance to nutrient depletion and stress. Viability was therefore reduced in *cwh47/ptc1* when compared to *cwh30/kic1.* A pattern is therefore emerging in which several genes are involved in regulating various pathways in *S. cerevisiae* that interconnect cell wall structure with the physiological status of the cell and the ability for the cell to adapt successfully to a constantly variable environment. The information already accumulated from

this study suggests that cell wall proteins such as Sed1p are expected to be least abundant in strains which are already known to have reduced ability to bind cell wall proteins and do not acquire resistance to Zymolyase, such as, *cwh48/kre6*.

Chapter 5

Quantification of Sed1p in *kre* strains of *S.cerevisae.*

5.0 Introduction

The cell wall of *Saccharomyces cerevisiae* is comprised of just four components; β1,6-glucan, β1,3-glucan, chitin and cell wall mannoproteins (CWPs). CWPs are covalently linked to the other components of the cell wall by mechanisms that are not yet fully understood (reviewed in Kapetyn *et al.*, 1999). It is thought that CWPs are covalently linked to the β1,3-component of the cell wall matrix by interactions involving chitin and β1,6-glucan (Kapetyn *et al.*, 1997). *Kre* strains of *S. cerevisiae* have reduced levels of β1,6-glucan and vary in their chitin content (Chapter 4). It would therefore be of interest to investigate whether this variation in composition affects the incorporation of CWPs into the cell wall. This chapter describes an investigation into the role of Sed1p in stationary phase *kre* strains. Sed1p is a GPI-CWP that is induced under conditions of stress and starvation (Shimoi *et al.*, 1998).

5.1 Involvement of mannoproteins in cell wall construction.

CWPs are believed to play an important role in protecting the cell and controlling permeability in response to environmental change (De Nobel *et al.*, 1998; Zlotnik *et al.*, 1984). Mannoproteins are categorised into three groups according to the way by which they are extracted from the cell wall. The extraction methods that have been used so far are by SDS, glucanase, reducing agents and NaOH (Moukadiri *et al.*, 1997; Kapetyn *et al.*, 1998; Mrša *et al.*, 1999).

5.1.1 SDS extractable cell wall proteins.

Research indicates that SDS extractable proteins are not covalently linked to structural polysaccharides but may be disulphide-linked to other proteins in the cell wall (Mrša *et al.*, 1999). A similar protein pattern found with SDS treated cell walls was discovered when extractions were carried out with DDT (Mrša *et al.*, 1999). Labelling cell wall proteins with a non-permeable biotinylation reagent, to eliminate intracellular contaminants, revealed there were nine SDS-soluble cell wall (SCW) proteins. When seven of these proteins were further analysed, three were found to have been already characterised as Cts1p (chitinase), Exg1p (exoglucanase) and Bgl2p (endoglucanase or transglucosidase) (Mrša *et al.*, 1997). All these proteins are believed to be involved in construction and renewal of the cell wall. Mrša *et al.* (1999) have concluded that the way by which cell wall proteins are anchored may reflect their function in cell wall maintenance and construction. Proteins non-covalently attached to cell wall polysaccharides may be required for the formation, maintenance and structure of the cell wall.

5.1.2 PIR cell wall proteins.

When SDS-extracted cell walls are incubated in 30 mM NaOH at 4°C, six proteins can be isolated by biotinylation (Mrša *et al.*, 1999). Most of these belong to a group of proteins from the *PIR* gene family, proteins which have a varying number of internal repeats at their N-terminal end. PIR-CWPs are highly *O*-glycosylated but do not carry a GPI sequence at the C terminus (Toh-E *et al.*, 1993). They are thought to be linked to the β1,3-glucan component of the cell wall by *O*-chains and may be a proportion of the small percentage (16%) of

proteins that remain attached to the cell wall after treatment with β1,6-glucanase (Kapteyn *et al.*, 1999).

5.1.3 Glucanase extractable cell wall proteins.

The remaining group of proteins are glucanase extractable. Glucanase extractable proteins remain in the cell wall after treatment with SDS but can be released by the cell wall degrading enzymes Zymolyase or laminarinase (Valentin *et al.*, 1984; Montijn *et al.*, 1994). As a consequence of glycosylation, these proteins often give rise to smeared bands. Of two enzymatic preparations used to extract the proteins, laminarinase was found to give rise to more distinct protein bands than Zymolyase. This is probably due to additional β1,6-glucanase activity present in laminarinase (Montijn, 1996).

Glucanase-extractable proteins in the cell wall share several common characteristics. They all have an *N*-terminal signal sequence, for entry into the ER, and C-termini containing a GPI addition sequence (Fig. 5.1.1). GPI protein precursors usually have two hydrophobic sequences. One is a signal sequence, which is necessary to enter the secretory pathway, and the other is a sequence that is cleaved off and replaced by a pre-formed GPI anchor (Ram *et al.*, 1998). The GPI anchor remains attached to the protein throughout the secretory pathway until it reaches the plasma membrane where it is then translocated to glycans in the cell wall by a transglycosylation reaction (Moukadiri *et al.*, 1997; Lu *et al.*, 1995). β1,6-glucan is thought to be part of the anchoring mechanism of CWPs to the cell wall (De Nobel *et al.*, 1994; Van der Vaart *et al.*, 1996). This study

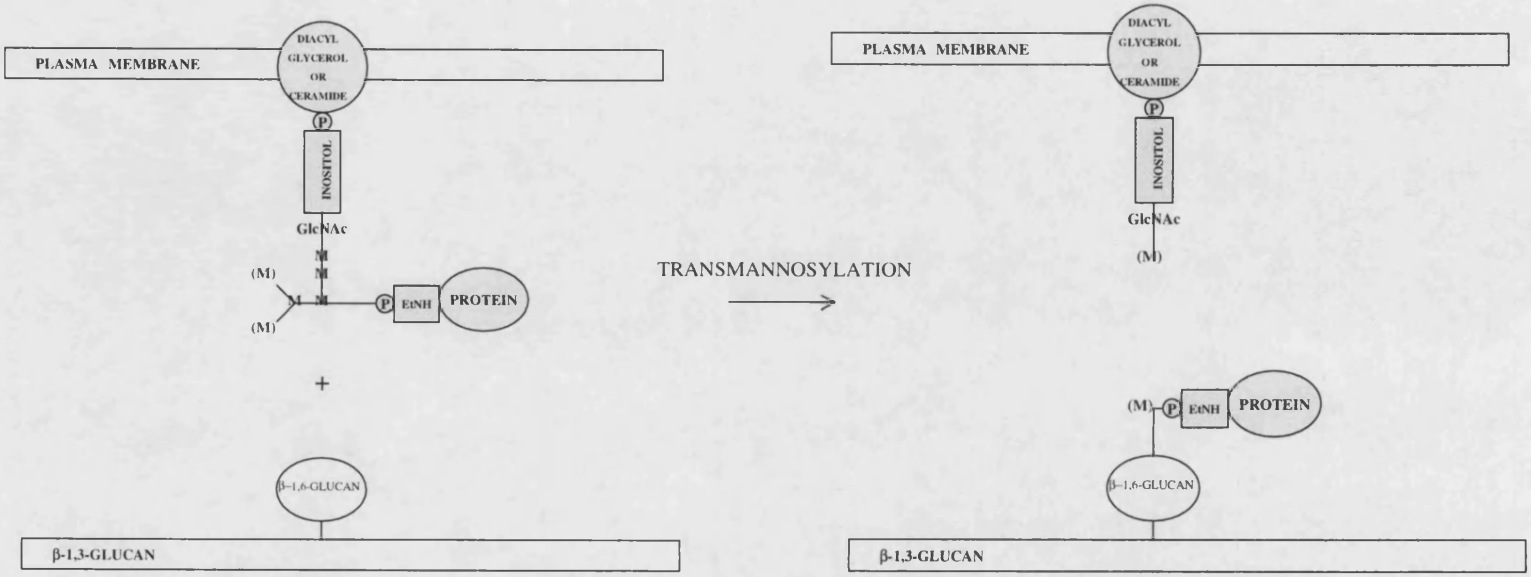


Figure 5.1.1. Schematic diagram of the linkage of the GPI-anchoring mechanism of glucanase-extractable cell wall proteins (from Mrša *et al*, 1999).

analyses what effect a reduction in β1,6-glucan would have on the quantity of a glucanase extractable CWP during stationary phase and further examines the relationship between reduced CWP levels and cell viability in long term stationary phase.

5.2 Function and characteristics of Sed1p.

In this study a cell wall protein containing a GPI-anchoring signal and associated with stationary phase was selected to monitor the abundance of GPI-CWPs in cell walls with reduced β1,6-glucan. Sed1p was selected to be epitope-tagged because it is abundant in the cell wall of stationary phase cells and is thought to offer cells additional protection in extreme environments (Shimoi *et al.*, 1998; Ezaki *et al.*, 1998).

5.2.1 Location and characteristics of SED1.

The *SED1* gene is located on the right arm of chromosome IV between base-pair co-ordinates 600787 to 601803. The length of the gene according to the *Saccharomyces* Genome Database (SGD) is approximately 1010 base-pairs. *SED1* is flanked either side on chromosome IV by *RAD55* and *PET100* (Coster *et al.*, 1995).

SED1 is a member of the *SED* family of genes, isolated because of their ability to suppress the *erd2* deletion (Hardwick *et al.*, 1992). The *ERD2* gene product is the receptor for a sorting signal, the C-terminal sequence HDEL, which allows proteins to be retained by the ER following modification in the Golgi complex (Munro and Pelham, 1987). Six genes belong to the *SED* family, *SED1* to *SED6*

(Table 5.2.1). *SED1*, encodes a cell surface glycoprotein, *SED2/SEC12* is required for the formation of ER-derived transport vesicles, *SED3/DPM1* is responsible for encoding a dolichol-phosphate-mannose involved in the synthesis of *N*-linked core oligosaccharides (Orlean *et al.*, 1988) and *SED4* encodes a protein related to Sec12p (Hardwick *et al.*, 1992). *SED5* encodes a t-SNARE (soluble NSF attachment protein receptor) required in ER to Golgi transport (Hardwick and Pelham, 1992). *SED6* carries out methylation of zymosterol as part of the ergosterol biosynthesis pathway (Hardwick and Pelham, 1994). All of these are membrane proteins that are associated with the secretory pathway.

Table 5.2.1. The *SED* gene family, function and gene products.

Gene	ORF/ Other names.	Gene product	Gene function	References
SED1	YDR077W	Putative cell surface glycoprotein.	Involved in stress protection.	Hardwick *et al.*, 1992. Shimoi *et al.*, 1998.
SED2	YNR026C *SEC12*	Guanine nucleotide exchange factor for Sar1p.	Required for recruitment of Sar1p and vesicle formation at the ER.	Hardwick *et al.*, 1992.
SED3	YPR183W *DPM1*	Dolichol phosphate mannose synthase.	Involved in the synthesis of *N*-linked core oligosaccharides.	Hardwick *et al.*, 1992.
SED4	YCR067C	Integral ER membrane protein.	Involved in vesicle formation at the ER.	Hardwick *et al.*, 1992.
SED5	YLR026C	Soluble NSF attachment protein receptor.	Required in ER to Golgi transport.	Hardwick and Pelham, 1992.
SED6	YML008C *ERG6*	Methylase.	Methylation of zymosterol as part of the ergosterol biosynthesis pathway.	Hardwick and Pelham ,1994.

Cells are unable to grow in the absence of the HDEL receptor. When Erd2p is depleted cells accumulate long strands of membrane in the cytoplasm that may be derived from the Golgi compartments (Semenza *et al.*, 1990). When the *SED*

proteins are over-expressed they suppress the *erd2* phenotype but still accumulate intracellular membranes (Hardwick *et al.*, 1992). In *erd2*Δ cells over-expressing *SED1*, the ER is normal but cells show an increased number of short membrane strands and vesicles in the cytoplasm (Hardwick *et al.*, 1992). The gene sequence of *SED1* is similar to YER150w and YJR151c which both express mucin like proteins.

Disruption of *SED1* results in increased sensitivity to Zymolyase and diamide (Ezaki *et al.*, 1998; Shimoi *et al.*, 1998). Increased sensitivity to Zymolyase indicates that Sed1p may offer defence against cell wall degrading and lytic enzymes. Increased sensitivity to diamide may indicate that Sed1p also protects the cell against oxidative stress. Yeast cells are normally resistant to diamide, a thiol-specific oxidant that oxidises glutathione. Only one other strain, *ROD1*, has been found to have this phenotype. *ROD1* encodes a stress-related protein which confers resistance to o-dinitrobenzene, calcium and zinc (Wu *et al.*, 1996). Resistance to o-dinitrobenzene is usually exhibited by over expressing glutathione S-transferase. Glutathione S-transferase is a membrane-bound protein that associates with the endoplasmic reticulum. Similarly to Sed1p, it is also induced in stationary phase (Choi *et al.*, 1998). So far it is known that *SED1* is induced by high concentrations of aluminium and zinc although it does not appear to offer the cell additional protection against these metal ions (Ezaki *et al.*, 1998). However, *SED1* does seem to offer protection against diamide, a chemical that increases the number of reactive oxygen species in cells. *SED1* was not induced by $CdCl_2$, $CoCl_2$, CrO_3 or $CuSO_4$ (Ezaki *et al.*, 1998).

SED1 may be expressed in stationary phase and in response to stress through STRE-mediated transcriptional activation (Shimoi *et al.*, 1998). Reduction of glucose concentration in stationary phase leads to the inactivation of protein kinase A through the RAS-cyclic AMP signal transduction pathway. The inactivation of protein kinase A activates the expression of stress-responsive element (STRE)-controlled genes. These genes have characteristic *cis* factors called STREs in their promoter regions (CCCCT or AGGGG). The promoter region of *SED1* contains both of these STREs, AGGGG at -84 bases from the translation initiation codon and CCCCT at -912. *SED1* also has a high codon bias index (0.7) which indicates that it is expressed at a high level (Shimoi *et al.*, 1998; Hardwick *et al.*, 1992; Bennetzen and Hall, 1982).

5.2.2 Characteristics and function of Sed1p.

The *SED1* gene encodes an abundant cell surface glycoprotein that is expressed moderately in exponential growth but increases expression 2.7 fold in stationary phase. Sed1p is composed of 338 amino acids and has a molecular weight of 34,429 Da (Fig. 5.2.1(A)) As in other CWPs, it is rich in threonine (29.3%) and serine (12.4%) and contains a signal sequence for the addition of a GPI anchor. Sed1p also contains four cysteine and six putative *N*-glycosylation sites which indicates that it is heavily glycosylated. Experimental evidence has revealed that the molecular mass of Sed1p can range from 100 to 810 kDa. It is heavily glycosylated by both *N*- and *O*-linked carbohydrates (Shimoi *et al.*, 1998).

The protein conformation of Sed1p is similar to a *Coccidioides* chitinase and human mucin (Fig.5.2.1(B)).The amino acid sequence is repeated in several areas

Figure 5.2.1. (A) Amino acid sequence of Sed1p and (B) pair-wise comparison *with* *Coccidioides immitis* chitinase (47.59% similarity).

A

1 *MKLSTVLLSAGLA*STTLAQFSNSTSASSTDVTSSSSISTSSGSVTITSSE 50

51 APESDNGTSTAAPTETSTEAPTTAIPTNGTSTEAPTTAIPTNGTSTEAPT 100

101 DTTTEAPTTALPTNGTSTEAPTDTTTEAPTTGLPTNGTTSAFPPTTSLPP 150

151 SNTTTTPPYNPSTDYTTDYTVVTEYTTYCPEPTTFTTNGKTYTVTEPTTL 200

201 TITDCPCTIEKPTTTSTTEYTVVTEYTTYCPEPTTFTTNGKTYTVTEPTT 250

251 LTITDCPCTIEKSEAPESSVPVTESKGTTTKETGVTTKQTTANPSLTVST 300

301 VVPVSS*SASSHSVVINSN**GANVVVPGALGLAGVAMLFL** 338

Italics indicate hydrophobic regions. Putative N-glycosylation sites and cysteine residues are underlined. The GPI-signal is double-underlined.

B

```
  1 ..............................MKLSTVLL.......SAGLA 13
                                      :..|||        ...:.
301 MAKYPSTFGGMMVWEATASENNKLGGLPYADIMKEVLLRCDPDPPTSTVT 350
             .            .            .            .
 14 STTLAQFSNSTSASSTDVT....SSSSISTSSGSVTITSSEAPESDNGTS 59
    ||| |  |. ||. ||.:.    |.|....|.:.|. .|. ...|..:||
351 STTSASTSTQTSSQSTTMETKTLSASTTPSSPSTVSPSSTMQTTSTGSTS 400
             .            .            .            .
 60 TAAPTETSTEAPTTAIPTNGTST........EAPTTAIPTNGTSTEAPTD 101
    ....|. | |:|.|.|.|.:.||        |:|.|.|.|.:.|||..|.
401 IETVTTRSQEPPSTTISTRSASTEPVTTRSQEPPSTTISTRSASTETVTT 450
             .            .            .            .
102 TTTEAPTTALPTNGTSTEAPT.............................. 122
     . |:|.|.:.| :.|||..|
451 RSQEPPSTTISTWSASTETSTSSQDSPSTTISTKSAPTGTVTTRSQDLPS 500
             .            .            .            .
123 ........DTTTEAPTT.............................GLPTNG 137
           :|.||.:||                               : .| :
501 TTISTRSPETETETATTKSQGSPSITLSTRSSSAETVSTRSQHSSSTTIS 550
             .            .            .            .
138 TTSAFPPT.......TSLPPS.............................. 151
    |.|| ..|        ||:|.|
551 TKSAPTETGTTSEHSTSMPVSTRSASTETVITRSQNSDSQSMTVSTRSPS 600
             .            .            .            .
152 ..NTTTTPPYNPSTDYTTDYTVVTEYTTY..CPEPTTFTTN.......GK 190
      ..|| .. .||..:.|. ..|....|   :..|||:.|.       :.
601 TESITTRSQGSPSETFSTKSVPVDTISTELPSQTPTTIITGTPSDPVSAP 650
             .            .            .            .
191 TYTVTEPTTLTITDCPCTIEKPTTTSTTEYTVVTEYTTYCPEPTTFTTNG 240
    | ||.. .|||:..:.:|.|..||..|   .:.|.|.| |  ||.||| .
651 TTTVPPNPTLTLAPSSSTTEDRTTITT...IITTSYVTVC..PTGFTTVT 695
             .            .            .            .
241 KTYTVT...EPTTLTITDCPCTIEKSEAPESSVP.VTESKGTTTKETGVT 286
     |||.|   |...|| |:.|.. ....:|::... || :.......|.:|
696 ITYTTTYCPETASLTPTQPPIPGAPAPPPDGWTTIVTVCPQCAPTPTTIT 745
             .            .            .            .
287 TKQTTANPSL.......TVSTVVPVSSSASSHSVVINSNGANVVVPGALG 329
     . .| .: |        .| |||||...: .:  |  |:::.|.... :
746 LTVPTRSAFLPARTETRPVVTVVPVPENPIKN..VKPSESGDFVTVTTAA 793
             .            .            .            .
330 LAGVAMLFL*.......................................... 339
     |.|.. :
794 PATVTKTLEYNNPVDSDVNVQPTGGSSPVEFEGSAMTVRSMDVVAKALIT 843
```

(Hardwick *et al.*, 1992). There is a 43 amino acid duplication and several short repeats (Fig. 5.2.1). These characteristics are often found in cell surface glycoproteins as revealed by the gene products of *KRE1* and *AGA1* (Hardwick *et al.*, 1992; Boone *et al.*, 1990; Roy *et al.*, 1991). Sed1p also contains 3 PEST-regions that usually contain regions rich in proline, glutamic acid, serine and threonine (P, E, S and T) (Coster *et al.*, 1995; Nash *et al.*, 1998; Vandenbol *et al.*, 1994). Clusters of positively charged amino acids usually flank these regions. PEST-regions are generally found in the C terminal region and are thought to be involved in protein degradation as shown by proteins, such as, Whi1p and Vth2p (Nash *et al.*, 1998).

The purpose of this work was to investigate the function of Sed1p in *kre* strains. This was to be determined by disrupting *SED1* and then constructing a fusion protein with either GFP or an HA-epitope tag.

Results and Discussion

5.3 Phylogeny of GPI proteins and polymorphism in the *SED1* gene.

In order to insert *SED1* into a suitable vector, primers were designed which would incorporate a restriction site at either end of the gene. A restriction map for *SED1* was first obtained from the SGD. Endonuleases *Eco*RI and *Hin*dIII were selected to flank either side of the *SED1* open reading frame as these enzymes did not cut the gene and were also available in the cloning site of the vector pUG36 (yEGFP3, N-FUS, URA3) (Chapter 2, 2.5.5). Using a software programme (PrimerCalc), forward and reverse primers, incorporating terminal restriction sites, were designed with melting temperatures of 55.4°C and 54.5°C

respectively. After several attempts these primers failed to give an adequate yield of PCR product using AR27 genomic DNA as a template. To ascertain if the genomic DNA was responsible for the low yield, genomic DNA from a wine producing strain, which was known to give a high yield of PCR product, was used as a template. Yield was still low but was improved by changing to a polymerase with increased activity (Bio-X-act, Bioline). Bands were faint but it was possible to see that the PCR product produced from this reaction gave bands that differed in size by approximately 100bp. Two more forward and reverse primers were designed with a higher GC content and melting temperature of 63.3°C and 65.4°C respectively (Table 5.3.1).

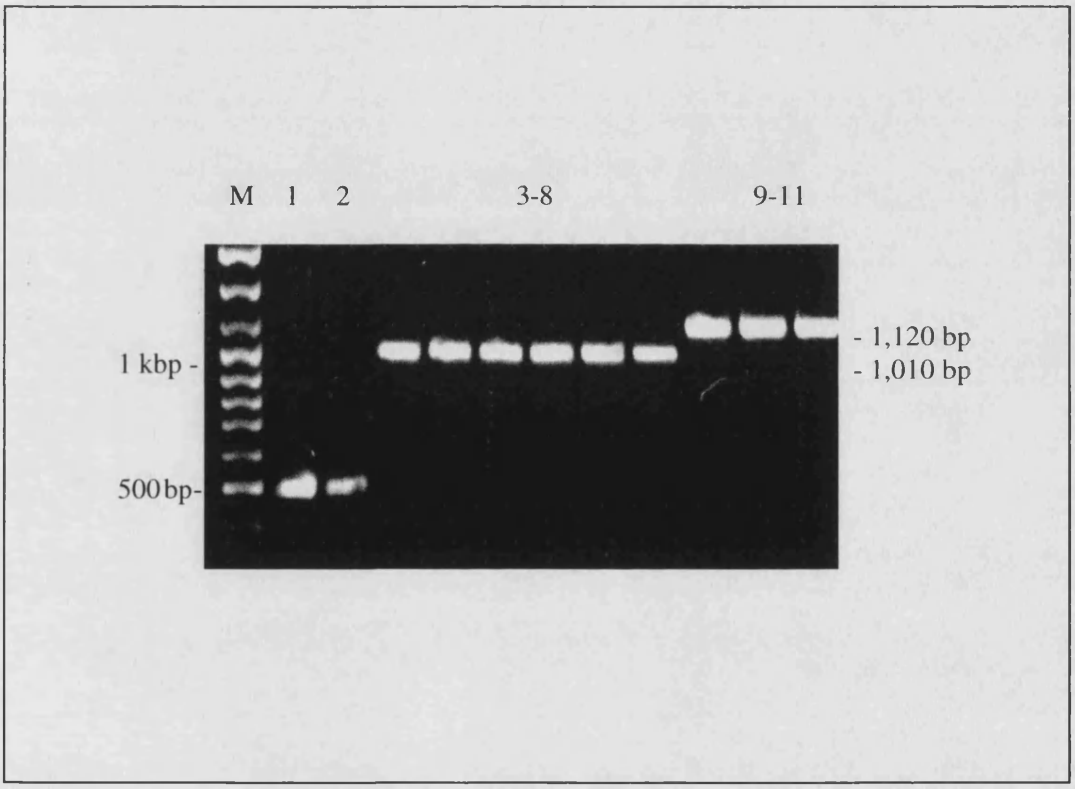
Table 5.3.1: Oligonucleotides used in PCR to amplify the *SED1* gene.

SEDf: 5' **GGAATTC**ATGAAATTATCAACTGTCCTATTATCTGCCGG 3' *Eco*R1 site in bold.
SEDr: 5' **CCCAAGCTT**TTATAAGAATAACATAGCAACACCAGCCAAACC 3' *Hind*III site in bold.

The repeated PCR using primers with a greater GC content gave a high yield product using a 50 µl PCR mix (Appendix 1) under the following PCR conditions; Initial denaturation (120 sec, 94°C) followed by 20 cycles of denaturation (30 sec, 94°C), annealing (30 sec, 54°C) and elongation (90 sec, 72°C). Then a final elongation (120 sec, 72°C) and cooled to 2°C.

The results confirmed the previous finding. PCR product of *SED1* amplified from genomic DNA of AR27 and the genomic DNA of the wine-fermenting strain differed in size by ~100 bp (Fig. 5.3.1). Further studies have revealed that

Figure 5.3.1: Polymorphism of *SED1* by PCR analysis. Primers for a non-coding region of AR27 (lane 1) produced the same size PCR product in a wine fermenting strain (lane 2). The *SED1* gene amplified in AR27 (lanes 3-8) and the same wine fermenting strain (lanes 9-11) gave DNA fragments that differ by over 100 bp. Electrophoresis was performed on a 1 % agarose gel.



polymorphism of *SED1* occurs frequently between strains of wine producing yeast (M Thangavelu, personal communication). This study uses the *SED1* gene amplified from AR27 and database sequencing information from strain FY1679. *SED1* amplified in FY1679 genomic DNA resulted in a PCR product the same size as that obtained from AR27 genomic DNA.

In a screen of the MIPS database a total of 51 GPI proteins have been found and categorised into families based on sequence homology (Caro *et al.*, 1997). Apart from all having a GPI anchor and a signal peptide they also contain serine and threonine rich regions, which are usually situated on the C-terminal half of the protein. These stretches are thought to become heavily glycosylated and have rod like conformations (Caro *et al.*, 1997). In some instances sequences are repeated (Marguet *et al.*, 1988). It is thought that this could be a means for the protein to span the cell wall (Caro *et al.*, 1997). The polymorphism found in *SED1* from different strains in this study could be caused by a variation in the number of repeated sequences. *SED1* contains a number of repeated serine and threonine regions.

An alternative theory is supported by genetic analysis carried out on brewery strains by Masneuf *et al.* (1998). Through karyotyping analyses and restriction fragment length polymorphism maps of PCR-amplified *MET2* gene fragments, a wine-making yeast and a cider-making yeast, were characterized for variation in

their nuclear and mitochondrial genomes. Sequence analysis of a part of two *MET2* gene alleles found that these two strains constituted hybrids between *S. cerevisiae* and *Saccharomyces bayanus*. The two hybrid strains also had different sequences of the *OLI1* gene. The sequence of the *OLI1* gene from the wine hybrid strain was nearly identical to the *S. cerevisiae* gene, whereas the *OLI1* gene of the cider hybrid strain was found to be equally divergent from both the parental strains, *S. bayanus* and *S. cerevisiae*.

Therefore polymorphic variation in the *SED1* gene in the wine making strain within this study could have been brought about by past hybridisation with other strains of *Saccharomyces sp.* This would also explain the variations found in other wine-making strains.

5.4 Reduced growth rate in *KRE6* with *SED1* gene disruption.

The *SED1* gene was disrupted in *kre* strains by small flanking homology PCR (SFH-PCR) using a method described by Wach *et al.* (1994) (Fig. 5.4.1). A DNA fragment containing the kanamycin resistant (*kanMX4*) module flanked by *SED1* primers was first amplified from *Not*I digested pFA6-*kanMX4* (Fig. 5.4.2). Oligonucleotide primers encoded a sequence of DNA from 5′ to 3′, primer S1 (or S2) has 40 nucleotides homologous to the 5′-site (or 3′-site) of the *S. cerevisiae* genomic target locus (*SED1*) followed by 18-19 nucleotides of *kanMX4* DNA (Table 5.4.1.). The PCR product of the *kanMX4* module flanked by target sequences was amplified under the following conditions using a 50 µl PCR mix (Appendix 1); Initial denaturation (120 sec, 94°C) followed by 20 cycles of

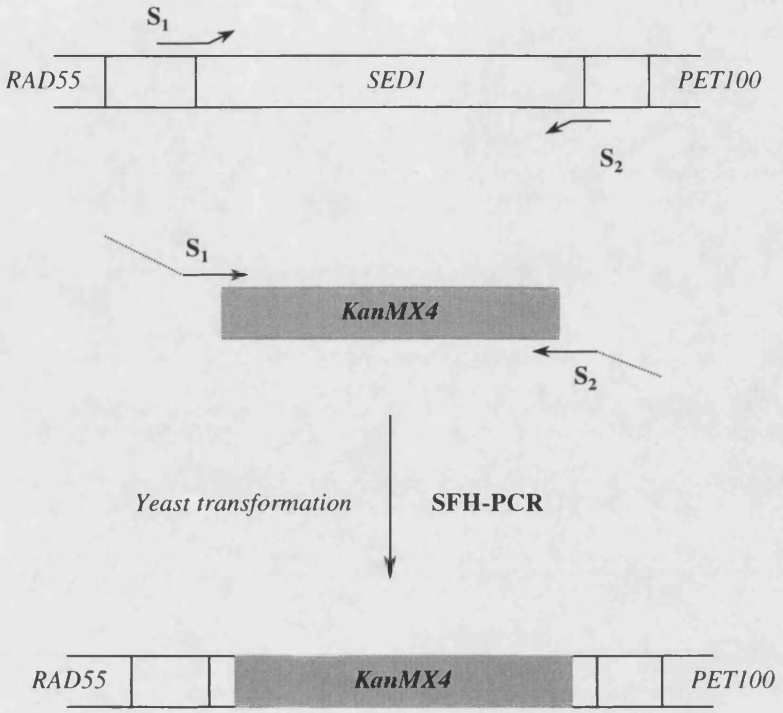


Figure 5.4.1. Schematic diagram of gene replacement of *SED1* with *KanMX4* by short flanking homology PCR (SFH-PCR). PCR product is generated from *KanMX4* using oligonucleotides with 20 nucleotides selected to bind to *KanMX4* and >35 nucleotides selected to bind to target DNA. The PCR product is then transformed into *S. cerevisiae* strains and by homologous recombination the *SED1* gene is disrupted with *KanMX4*.

denaturation (30 sec, 94°C), annealing (30 sec, 47°C) and elongation (90 sec, 72°C). Then a final elongation (120 sec, 72°C) and cooled to 2°C.

The PCR amplified product was transformed into haploid AR27 and *kre* strains (*cwh13/erd1, cwh30/kic1, cwh41/gls1, cwh47/ptc1 and cwh48/kre6*) using the LiAc method described by Gietz and Schiestl (1995). These were then selected for kanamycin resistance by incubating for three days at 28°C on solid YPD media containing geneticin (G418) 200-250 mg/L. The transformation frequency pattern with the *kanMX4* PCR product was similar in the *kre* strains as with transformations involving plasmid YPB1-ADHpt (Chapter 4, 4.7). AR27, *cwh13/erd1* and *cwh41/gls1* gave the highest number of transformants (~100 to 1000). The remaining *kre* strains, *cwh30/kic1*, *cwh47/ptc1* and *cwh48/kre6*, required a longer incubation period and gave relatively few transformants (<10).

Table 5.4.1: Oligonucleotide used in gene disruptions.

SED1 gene disruption.

S1:

5' TGTCCTATTATCTGCCGGTTTAGCCTCGACTACTTTGGCC**CGTACGCTGCAGGTCGAC** 3'

S2:

5' AACATAGCAACACCAGCCAAACCTAAAGCACCTGGAACGA**ATCGATGAATTCGAGCTCG** 3'

Kanamycin sequence in bold.

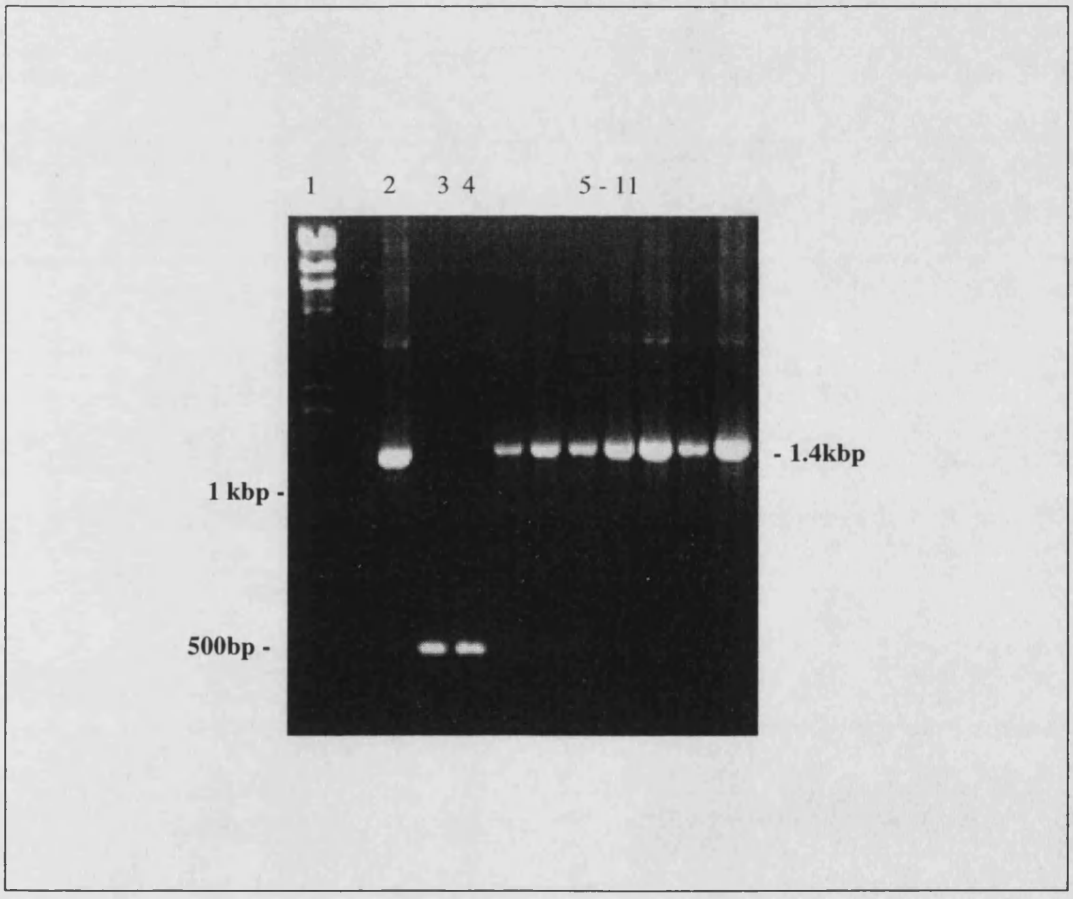
KanMX4 location.

A1: 5' TACGAAAGAGGAGAAGGG 3'

KAN: 5' CAGTTCTCACATCACATCC 3'

Initially, cells transformed with *kanMX4* produce two types of colonies. A few large colonies (3-4 mm diameter) are found amongst several smaller colonies (0.2-2 mm) in diameter. The smaller colonies are thought to be of cells that

Figure 5.4.2. Disruption of *SED1*. Amplification of a DNA fragment (*kanMX4*) containing the kanamycin resistant gene flanked by primers from the target gene was first amplified from *Not*1 digested pFA6-*kanMX4*. Lane 1, λ DNA used for quantification; Lane 2, control *kanMX4* DNA; Lanes 3 and 4, genomic non-coding DNA; Lanes 5-11; duplicates of *kanMX4* with target gene termini.

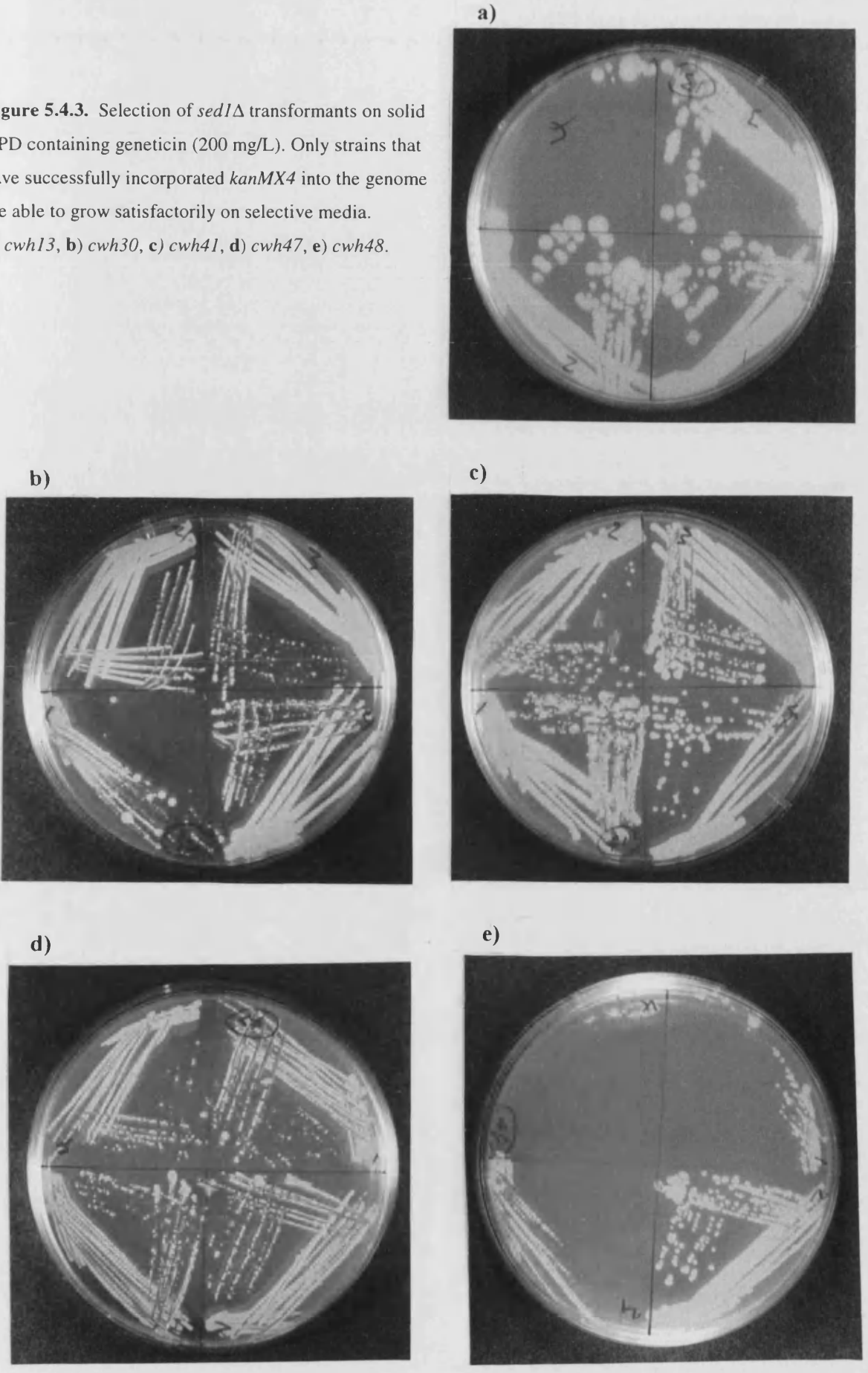


contain non-integrated *kanMX4* DNA. It is thought that these cells can produce enough aminoglycoside-phosphotransferase to inactivate geneticin G418 for a number of cell divisions (Wach *et al.*, 1994).

To segregate potential transformants from those which contained non-integrated *kanMX*, colonies that were above 3-4 mm in diameter were streaked onto solid media containing geneticin (G418) 200-250 mg/L. Those that contained integrated *kanMX* gave strong growth and were capable of forming colonies (Fig. 5.4.3). The strains (*cwh30, cwh47, cwh48*) that required longer incubation following the transformation also grew slower when they were streaked onto selective media; *cwh13* and *cwh41* gave stronger growth than *cwh30* and *cwh47* (Fig. 5.4.3). This could be attributed to decreased fitness in the strain due to the *SED1* disruption. Four colonies from each transformed strain were streaked onto selective media. The colonies that gave strong growth were further assessed by PCR. The *kanMX4* DNA in transformants was located by PCR analysis of lysed cells using an oligonucleotide that would amplify a region of DNA upstream of *SED1* and an oligonucleotide corresponding to a region of DNA from within the *kanMX4* module (Table 5.4.1).

Those that had successfully incorporated the *kanMX4* module produced a gene product of ~650 bp (Fig. 5.4.4). Sequence analysis of this region confirmed that

Figure 5.4.3. Selection of *sed1*Δ transformants on solid YPD containing geneticin (200 mg/L). Only strains that have successfully incorporated *kanMX4* into the genome are able to grow satisfactorily on selective media.
a) *cwh13*, **b**) *cwh30*, **c**) *cwh41*, **d**) *cwh47*, **e**) *cwh48*.

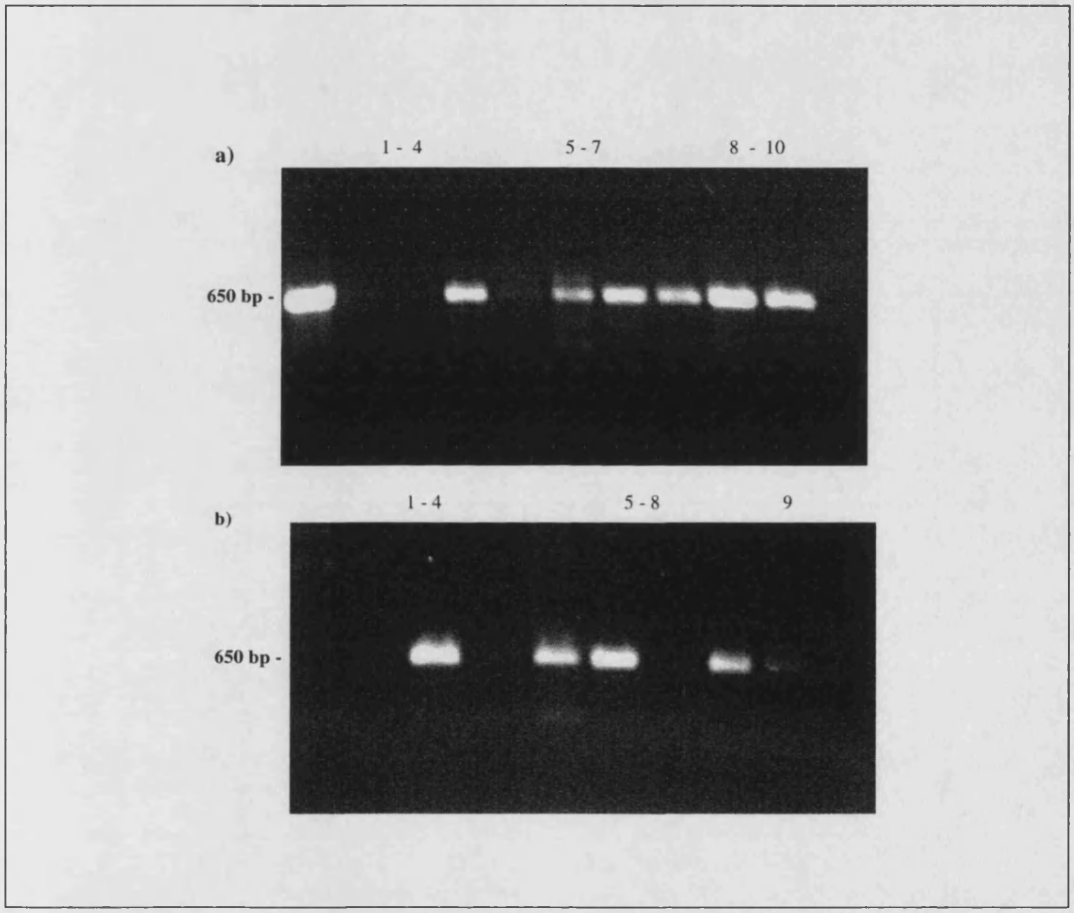


the DNA product contained the *kanMX* gene (Appendix 3). Empty lanes represent colonies that had acquired geneticin resistance but may have incorporated the gene into a different position in the genome.

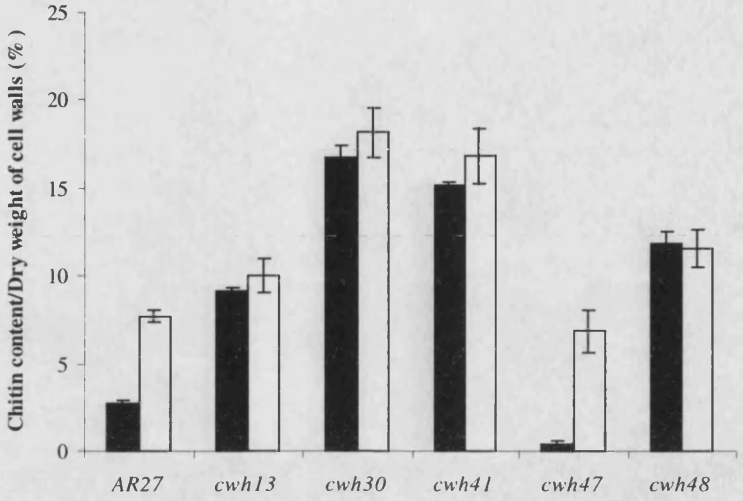
Strains with the *SED1* disruption were then further analysed for phenotypic alterations. To assess the effect of losing an abundant CWP from strains that already had a defective cell wall, they were assessed for various stress responses such as Zymolyase resistance, sensitivity to Calcofluor White, diamide and copper ions. Alterations in cell wall composition were also monitored by measuring chitin and protein levels (Fig. 5.4.5).

Only two of the strains with a disruption in the *SED1* gene, AR27 and *cwh47/ptc1*, show a significant difference in cell wall chitin content following two weeks incubation (Fig. 5.4.5 (a)). Chitin levels are lower in *cwh47/ptc1* than in the other *kre* strains. This could be because daughter cells do not separate from the mother cell. The cells therefore would have a lower number of budding scars than in the other *kre* strains. By a similar manner to other CWPs, Sed1p is incorporated into the cell wall during M phase of the cell cycle (De Nobel *et al.*, 1991; Caro *et al.*, 1998). This could imply that if a major stress protectant CWP is absent from the cell wall, chitin deposition is increased to compensate for the defect. Alternatively, the loss of a major CWP that offers protection to the cell wall could increase the risk of damage and cause the cell to compensate by operating a cell wall repair mechanism. Interestingly, there was no significant

Figure 5.4.4: *KanMX* fragment detected by PCR analysis in strains transformed with kanMX4 gene fragment. **a)** Lanes: 1-4 , AR27; 5-7 *cwh13*; 8-10, *cwh30*. **b)** Lanes: 1-4, *cwh41*; 5-8, *cwh47*; 9, *cwh48*. Electrophoresis was performed on 1 % agarose gels.



a)



b)

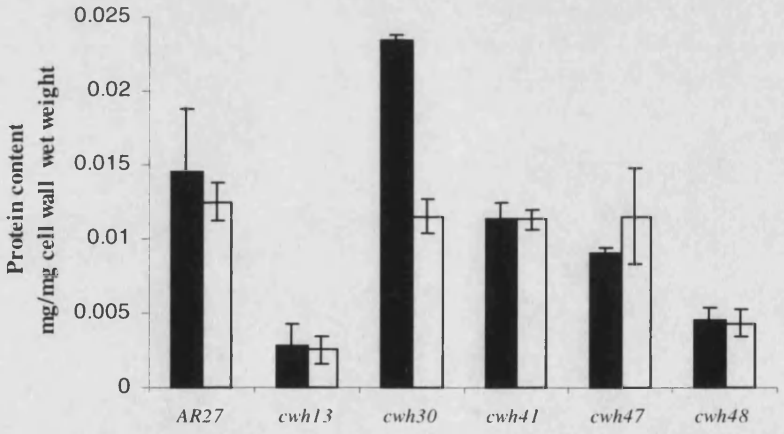


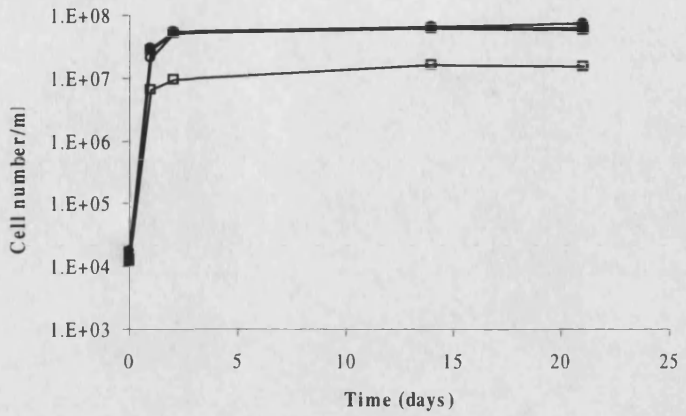
Figure 5.4.5. The influence of Sed1p on chitin and cell wall protein content in *kre* strains of *S. cerevisiae*. Strains were grown for two weeks in SC media containing 2% (w/v) glucose. Cell walls were then isolated by glass bead cell disruption. For chitin quantification, cell walls were degraded by overnight hydrolysis with HCl at 100°C. Chitin content was determined from glucosamine measurements. Protein quantification on intact cell walls was determined by NaOH hydrolysis. *SED1*$^{+}$ strains are represented by filled columns (■), *sed1* strains are represented by open columns (□). Mean ± SEM.

difference in the protein levels of any of the *kre* strains except for in *cwh30/kic1* (Fig. 5.4.5 (b)). *cwh30/kic1* gave a substantial reduction in cell wall proteins in the *sed1* strain (from 0.023 down to 0.012 mg/mg cell wall (wet wt)). This implies that *SED1* is induced in this strain and that a substantial proportion of the protein in the cell wall of this strain is probably Sed1p. This increases the evidence that *cwh30/kic1* is in a stress induced state when compared to the wild-type or other *kre* strains.

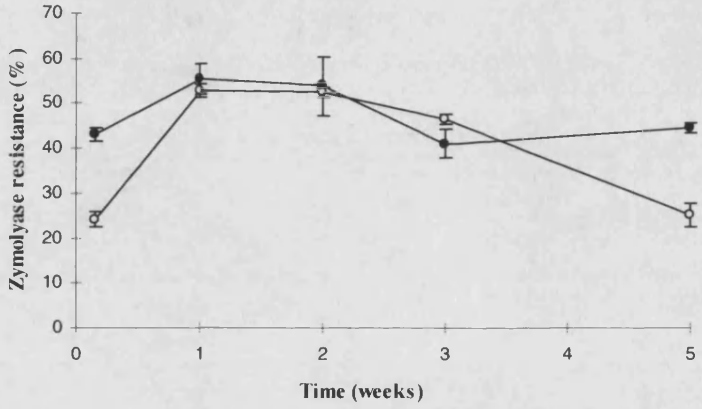
The most obvious physical change attributed to the loss of Sed1p, was in *cwh48/kre6* which showed increased flocculation and a reduction in yield. It also had a reduced resistance to Zymolyase, in the early stages of stationary phase, possibly as a consequence of the flocculating phenotype (Fig. 5.4.6).

The *cwh48/kre6* phenotype allows proteins to be secreted into the media (Roemer *et al.*, 1994). Overexpression of Sed1p suppressed the defect of *erd2* mutants, a gene involved in retaining proteins within the ER following modification in the Golgi complex (Munro and Pelham, 1987). Kre6p resides in the Golgi body where it is involved in protein modification (Roemer *et al.*, 1994). This mutation appears to be emphasised by the absence of Sed1p. This may indicate that Sed1p not only acts as a stress protectant when incorporated into the cell wall but additionally has a supportive role in the retention of proteins within the ER.

a)



b)



c)

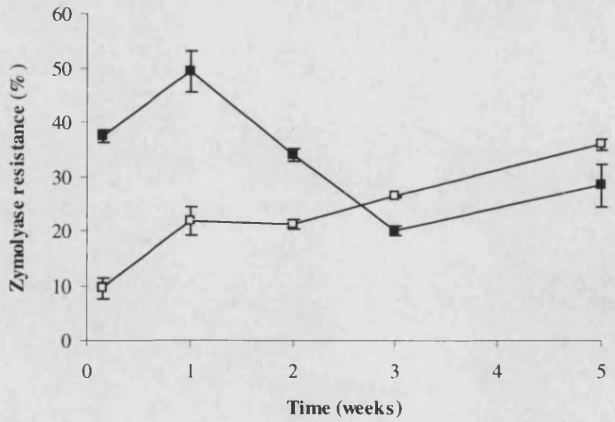


Figure 5.4.6. Influence of Sed1p on cell number/ml and Zymolyase resistance in *cwh48/kre6*. **a**) Cell number/ml of *cwh48/kre6Δsed1* (□) was compared to *cwh48/kre6* (■) and AR27 with (●) and without (○) the *SED1* gene. Strains were grown in SC media containing 2% (w/v) glucose. b) Zymolyase resistance in AR27 (●) and AR27*sed1*Δ (○) and c) *cwh48/kre6* (■) and *cwh48/kre6 sed1*Δ (□). Strains were grown in YPD with 0.3 % (w/v) glucose. Mean ± SEM.

Preliminary findings of Zymolyase resistance in the AR27*sed1Δ* parental strain are similar to published results (Shimoi *et al.*, 1998; Caro *et al.*, 1998). AR27*sed1Δ* was found to have increased sensitivity to Zymolyase during the first 24 hours of growth (Shimoi *et al.*, 1998) (Fig 4.4.6(b)). It was then as resistant as the parental strain during early stationary phase with sensitivity gradually returning as stationary phase progressed.

SED1 disruptants previously found to be sensitive to Zymolyase were from a 48 hour culture grown in YPD containing 2% (w/v) glucose (Shimoi *et al.*, 1998). By the interpretation presented in this study these cells were non-dividing or entering the diauxic shift and not cells in stationary phase. Another recent study involving GPI proteins has not found a Zymolyase sensitive phenotype in *sed1*$^-$ strains but in this case resistance was assessed following two weeks growth (Caro *et al.*, 1998). These results are similar to those found within this study. However in *kre6 sed1* disruptants, sensitivity to Zymolyase continues for over 14 days growth (Fig. 5.4.6(c)). This probably implies that the degree of resistance to lytic enzymes offered by Sed1p differs depending on the nature of the mutation. Budded slow growing populations with a cell wall composition similar to exponentially dividing cells will probably show a higher sensitivity to Zymolyase than those that have already adopted stationary phase characteristics. An assay of cell wall proteins extracted with sodium hydroxide also revealed that *cwh48/kre6* had reduced protein levels in the cell wall when compared to the wild type. The *cwh48/kre6* strain was also the least viable in long term stationary phase (Chap 4, section 4.2.4).

5.5 Characterization and quantification of Sed1p in *kre* strains during stationary phase.

Sed1p is a glucanase-extracted cell wall protein which contains a GPI anchor (van der Vaart *et al.*, 1996; Shimoi *et al.*, 1998). It is composed of 338 amino acids and has a calculated molecular mass of 34,429 Da. The primary structure of Sed1p is highly glycosylated by *N*- and *O*-linked sugars (Shimoi *et al.*, 1998). The protein has been isolated and purified from *mnn9* cells and wild-type cells and analysed by SDS-page after treatment with PNGase F to eliminate *N*-linked sugars from proteins (Shimoi *et al.*, 1998). It was subsequently discovered that Sed1p from wild-type cells migrated slower than that from *mnn9* cells. In mnn9 mutants a defect occurs in the secretory pathway which causes the outer chains of N-linked sugars to become truncated (Ballou *et al.*, 1980). The protein produced a smeared band on SDS-page in both strains. The estimated molecular weight was decreased following PNGase treatment in both strains. In wild-type cells the decrease was from 360-810 kDa to 110-230 kDa while in *mnn9* cells the protein was reduced from 150-270 kDa to 100-190 kDa (Shimoi *et al.*, 1998).

Several attempts were made within this study to create a Sed1p fusion protein containing either a Green Fluorescent Protein (GFP) or haemaglutinin (HA) epitope tag. The first attempt to make a GFP fusion protein was by incorporating *SED1* into a specifically designed yEGFP fusion vector system. The *SED1* region was amplified from genomic DNA extracted from AR27 with primers containing *Eco*RI/*Hin*dIII restriction sites (see section 5.3). *Kpn*I digestion indicated that the PCR product was *SED1*. The *Eco*RI/*Hin*dIII digested DNA fragment containing *SED1* was inserted into *Eco*RI/*Hin*dIII digested pUG36 (yEGFP3, N-FUS,

URA3) (Fig. 5.5.1) and ligated to give a sequence that would eventually encode a GFP protein fused to the N-terminus end of Sed1p. The GPI anchor required for cell wall attachment is located at the C-terminus end of Sed1p. The ligated plasmid was transformed and cloned into *E. coli*. Insertion of the DNA fragment into pUG36 was confirmed by PCR analysis on lysed cells of *E. coli* using a 50 µl PCR mix (Appendix 1) under the following conditions; Initial denaturation (120 sec, 94°C) followed by 30 cycles of denaturation (30 sec, 94°C), annealing (30 sec, 47°C) and elongation (90 sec, 72°C). Then a final elongation (120 sec, 72°C) and cooled to 2°C.

PCR analysis confirmed that *SED1* had been successfully inserted into pUG36 (Fig. 5.5.1). The plasmid was cloned and extracted from *E. coli* and then transformed into AR27 and *kre* strains (*cwh13/erd1, cwh30/kic1, cwh41/pls1, cwh47/ptc1* and *cwh48/kre6*) by a LiAc method described by Gietz and Schiestl (1995). Although the strains were successfully transformed with the plasmid, further investigations using this Sed1p-GFP construct were discontinued because the method failed to consider the hydrophobic signal sequence that is needed to modify the protein through the secretory pathway. The GFP-tag would have either been spliced off or could have obstructed the passage of the protein through the ER.

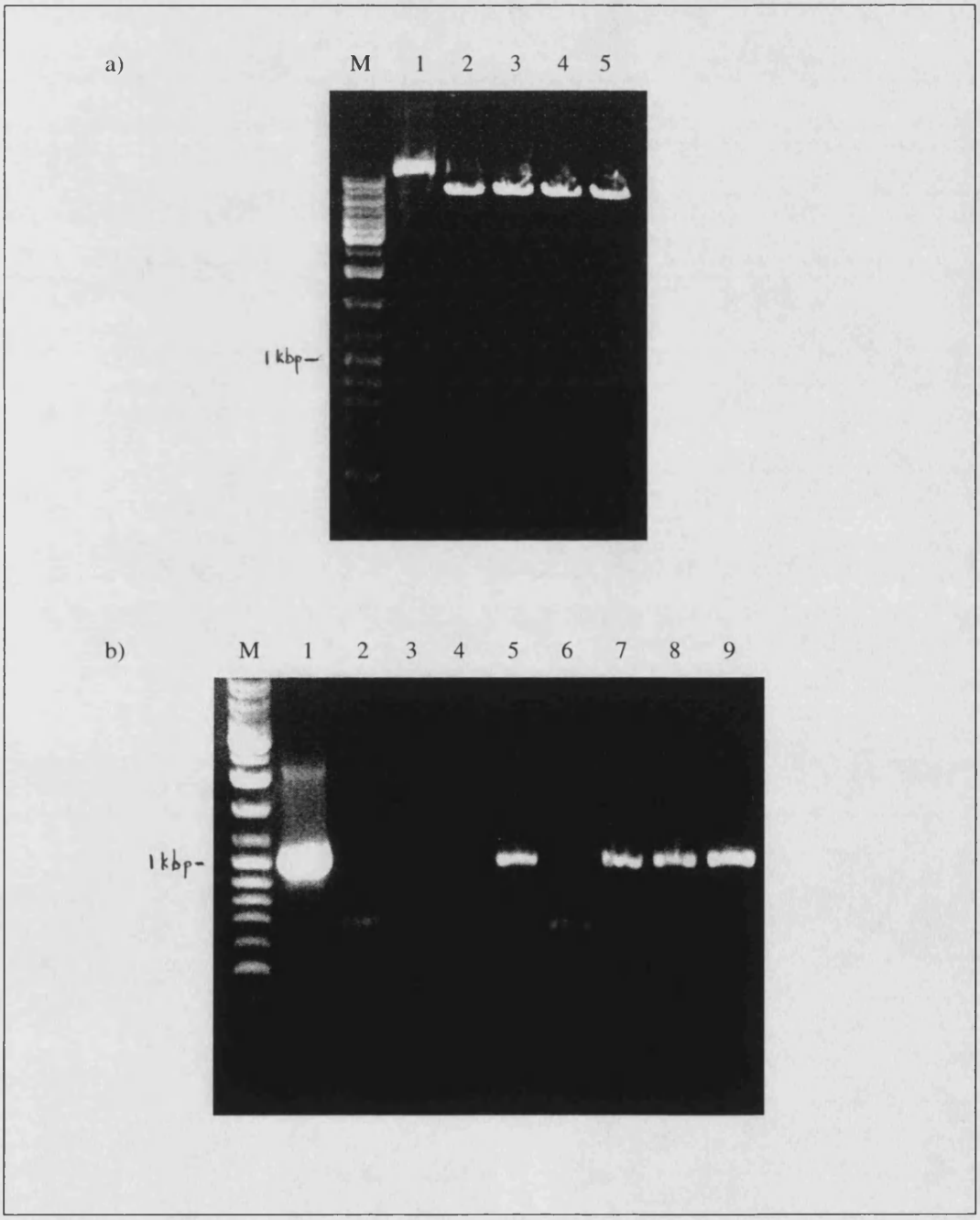
An alternative technique was attempted, to insert an epitope tag after the signal sequence using a PCR procedure involving gene splicing by overlap extension in combination with information from a successfully epitope tagged Sed1p

Figure 5.5.1.

a) Restriction endonuclease digestion of pUG36 (yEGFP3, N-FUS, URA3) with *Eco*RI/*Hin*dIII. Lane 1, Undigested pUG36 control; Lane 2, pUG36 digested with *Eco*RI; Lane 3, pUG36 digested with *Hin*dIII; Lane 4 and 5, pUG36 digested with *Eco*RI and *Hin*dIII .

b) Detection of *SED1* insert in transformed *E. coli* cells. Lane 1, *SED1* amplified from genomic AR27. Lanes 2-9, *E. coli* cells transformed with pUG36 and *SED1* insert. Empty lanes indicate an unsuccessful ligation.

Electrophoresis was performed on 1 % agarose gels.



experiment (Ho *et al.*, 1989; Ram *et al.*, 1998; Shimoi *et al.*, 1998). Mutagenesis by overlap extension involves generating two PCR products from a fragment of DNA with complementary overlap extensions. When the two PCR products are combined, the overlap extensions fuse to create a single fragment of DNA with an insert containing annealed extensions (Fig. 5.5.2). The overlap extension in this case was a sequence encoding the influenza virus HA 12CA5 epitope flanked by two restriction enzyme sites (Table 5.5.1).

Table 5.5.1. Oligonucleotides used to construct an HA-epitope tagged fusion protein.

A_1) SEDf: 5' **GGAATTC**ATGAAATTATCAACTGTCCTATTATCTGCCGG 3'
*Eco*RI site in bold.

C_1) SEDr: 5' **CCCAAGCTT**TTATAAGAATAACATAGCAACACCAGCCAAACC 3'
*Hin*dIII site in bold.

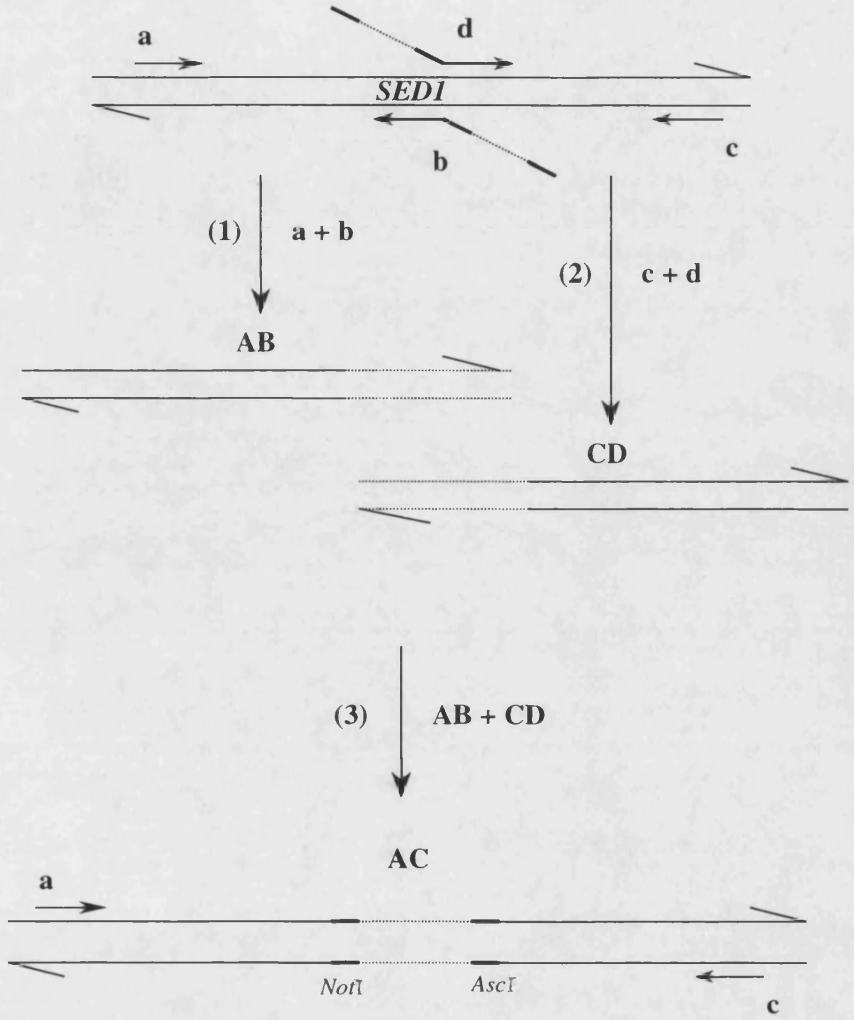
A_2) FragEco*RI*f: 5' AAC**GAATTC**GAATCCTTGGAAGAATA 3'
*Eco*RI site in bold.

C_2) FragBam*HI*r: 5' GCT**GGATC**CTCATAGCTTATCAAAG 3'
*Bam*HI site in bold.

D) HAfSED1:
5' TACCCATACGACGTCCCAGACTACGCT**GGCGCGCC**CAATTTTCCAACAGTACATCTGCT 3'
HA epitope sequence underlined. *Asc*I restriction site in bold.

B) HArSED1:
5' AGCGTAGTCTGGGACGTCGTATGGGTA**GCGGCCGC**GGCCAAAGTAGTCGAGGCTAAACC 3'
HA epitope sequence underlined. *Not*I restriction site in bold.

An attempt was made to insert the epitope tag in the *SED1* open reading frame and also in an *Eco*RI/*Bam*HI digested 3.3 kb fragment containing *SED1*, the promoter regions and STREs sites associated with *SED1* (Fig. 5.5.3). Restriction sites for the endonucleases *Not*I and *Asc*I were selected to flank the HA-epitope sequence because they did not already occur in the 3.3 kb *Eco*RI/*Bam*HI fragment containing *SED1*.



HA-epitope tag inserted into *SED1*

Figure 5.5.2 . Schematic diagram of site-directed insertion of HA-epitope into *SED1* by overlap extension using PCR products. Oligonucleotides are indicated by lower case. PCR products generated by the corresponding oligonucleotides are represented by uppercase. The final product is an HA epitope sequence inserted into *SED1* and flanked by two restriction sites (*Not*I and *Asc*I).

PCRs were performed on genomic DNA from AR27 using oligonucleotide combinations of A_1 + B, C_1 + D, A_2 + B and C_2 + D and 50µl PCR mix (Appendix 1) under the following conditions; initial denaturation (120 sec, 94°C) followed by 30 cycles of denaturation (30 sec, 94°C), annealing (30 sec, 47°C) and elongation (90 sec, 72°C). Then a final elongation (120 sec, 72°C) and cooled to 2°C.

This resulted in eight separate gene products. To obtain two HA-epitope tagged DNA fragments the PCR products of A_1 + B and C_1 + D were used as the template in one reaction (1) and the PCR products of A_2 + B and C_2 + D were used as a template in a separate reaction (2). The oligonucleotides A_1 and C_1 were used as primers in the first reaction (1) and A_2 and C_2 were used as primers for the second reaction (2). Both reactions use a 50 µl PCR mix (Appendix 1) under the following conditions; Initial denaturation (120 sec, 94°C) followed by 10 cycles of denaturation (15 sec, 94°C), annealing (30 sec, 50°C) and elongation (120 sec, 72°C) followed by 15 cycles of denaturation (15 sec, 94°C), annealing (30 sec, 55°C) and elongation (130 sec, 72°C) then a final elongation (5min, 72°C) and cooled to 2°C.

By using the above PCR conditions and Expand polymerase preparation (which contains *Taq* and *Pwo* polymerases) two gene products were obtained of the correct size (Fig. 5.5.4). One PCR product was ~1.1 kb which was the anticipated size of the *Eco*RI/*Hin*dIII fragment with *SED1*::HA. The other gene product was ~3.3 kb which was the anticipated size of the *Eco*R1/*Bam*H1 fragment containing the promoter region and *SED1*::HA.

Figure 5.5.3. Fragment of DNA (co-ordinates 598880 to 602740) from chromosome IV containing *SED1* and promoter region. Relevant restriction sites and stress responsive elements are indicated in bold. DNA inserted into plasmid pRS416-HA::*SED1* was taken from the restriction sites *Eco*RI to *Bam*HI (Shimoi *et al.*, 1998).

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1 TAGTCTGCGA TCATCTGTGT ACACTAAGTA AAAGAGGCGG CGGAATGATA

51 GATAAAACTA TACACGAGTT AAAATGTCGA CATTTGATAT TAATTTTTAC

101 GGTAATGACC AAATACACAC ATTCCACGGG TTCGACAATA ATAGTTTTAA

*Eco*RI

151 ATGACTGTAT GAACACCGCC TTCCAATCAA AC**GAATTC**GA ATCCTTGGAA

201 GAATACTACG AAATTTTAGA CGACGGATCT AACTTCTTTG TCAACTCTAA

251 CAACGAAAGG CGTAAGAACA ATGTACATAT ATTGAAAAGC GCGCTCGTTG

301 CCAATATCGC TATGGGAAGC AAGGATTCCA CTTGGGAAGT ATTTCTAAGA

351 GACAGGATCG GCCTTTTTAG GGATTGGAAT GAACAAGTGG ACGAAACCGT

401 TTTCGTGAAA AGCAAAAGAG TGAAGGCGTC ATCCTCGCAG AGTAACGAGG

451 GATGTACTAC CATCAAAGAA ATGAGAATAA ACAAACGAAA CTTTGAAAAC

501 TTGAGAATAG CTATTGTTTT TAATTTACAC GGCGAAGATA GAAAAAGAGA

551 AGGACGAAAT TTAAAGCGGT CAAGAAGCAG CGACGATCGT AACTATATTG

601 TCAAATTTGA TTTTGATAAA GCAACAGGTC AACTCCGCGA TATAATTGAT

651 CTGAAACCTG ATACTGCTAA TATTGCCTCA TTTCCAACAT TATCAACAAG

701 CAGCAGCAGT TGCTCACAAG TGTTTAACAA TATTGACTCC AATGATAATC

751 CATTACCAAA TGCGGAGGGA AAGGAGGAGA TAATTTATGA TAGTGAAGGT

← *RAD55*

801 TAATAGTAAA ATAAAAAAAC CGAAAACCGA AAACAATAAA AAATGGAAAA

851 ACGACAACAT TCCACCCAAC AACTACAAAG AAAAGTTAAG GGTAATTTTA

901 CCTATTTAGG ATTTTAATCT GTTGGAGTTA AGGTGAATAC GTTTTTCCAT

STRE

951 ATTGGGGTAT GCAGCTCGAA CCTAAAGTGG TATGTACACA T**CCCCT**CAAG

1001 CACACCCATT ACCCTTATAG GATTAATGTA AGCAACAGCT TACACGGAAT

1051 TGGAAATACT ATTCAACGAT CCATGCATCT GCCAGATTCG GACATGCATA

1101 TTCCCCAATT GGATATAGAA AATTAACGTA AGGCAGTATC TTTTCACAAT

1151 GTACTTGCAA CGCGGCGACT TAAAGTTGAA GTACAACCTG CAGCAGCGGC

1201 TTTTTGTACG GTACGCCAAA CTGTCAATGG ATAATATTGC GTAGACCGAA

1251 AAAGGTAATC CTCAACACTA CCCGTGGTGG ATGACCTAAA GCAGTAATAT

*Hin*dIII

1301 TGGTTGGAAT TATCTCCCAG ACGGCACCGT CTCCCCGAGA **AAGCTT**AGCC

1351 CCGAGGTCTA CCTTCCATAC ACCACTGATT GCTCCACGTC ATGCGGCCTT

1401 CTTTCGAGGA CAAAAAGGCA TATATCGCTA AAATTAGCCA TCAGAACCGT

1451 TATTGTTATT ATATTTTCAT TACGAAAGAG GAGAGGGCCC AGCGCGCCAG

1501 AGCACACACG GTCATTGATT ACTTTATTTG GCTAAAGATC CATCCCTTCT

1551 CGATGTCATC TCTTTCCATT CTTGTGTATT TTTGATTGAA AATGATTTTT

1601 TGTCCACTAA TTTCTAAAAA TAAGACAAAA AGCCTTTAAG CAGTTTTTCA

1651 TCCATTTTAC TACGGTAAAA TGAATTAGTA CGGTATGGCT CCCAGTCGCA

STRE

1701 TTATTTTTAG ATTGGCCGT**A GGGG**CTGGGG TAGAACTAGA GTAAGGAACA

1751 TTGCTCTGCC CTCTTTTGAA CTGTCATATA AATACCTGAC CTATTTTATT

1801 CTCCATTATC GTATTATCTC ACCTCTCTTT TTCTATTCTC TTGTAATTAT

1851 TGATTTATAG TCGTAACTAC AAAGACAAGC AAAATAAAAT ACGTTCGCTC

SED1→

1901 TATTAAGATG AAATTATCAA CTGTCCTATT ATCTGCCGGT TTAGCCTCGA

HA epitope tag

1951 CTACTTTGGC C↓CAATTTTCC AACAGTACAT CTGCTTCTTC CACCGATGTC

2001 ACTTCCTCCT CTTCCATCTC CACTTCCTCT GGCTCAGTAA CTATCACATC

2051 TTCTGAAGCT CCAGAATCCG ACAACGGTAC CAGCACAGCT GCACCAACTG

2101 AAACCTCAAC AGAGGCTCCA ACCACTGCTA TCCCAACTAA CGGTACCTCT

2151 ACTGAAGCTC CAACCACTGC TATCCCAACT AACGGTACCT CTACTGAAGC

2201 TCCAACTGAT ACTACTACTG AAGCTCCAAC CACCGCTCTT CCAACTAACG

2251 GTACTTCTAC TGAAGCTCCA ACTGATACTA CTACTGAAGC TCCAACCACC

2301 GGTCTTCCAA CCAACGGTAC CACTTCAGCT TTCCCACCAA CTACATCTTT

2351 GCCACCAAGC AACACTACCA CCACTCCTCC TTACAACCCA TCTACTGACT

2401 ACACCACTGA CTACACTGTA GTCACTGAAT ATACTACTTA CTGTCCAGAA

2451 CCAACCACTT TCACCACAAA CGGTAAGACT TACACCGTCA CTGAACCAAC

2501 CACATTGACT ATCACTGACT GTCCATGCAC CATTGAAAAG CCAACAACCA

2551 CATCAACCAC CGAATACACT GTAGTCACTG AGTACACTAC TTACTGTCCA

2601 GAACCAACCA CTTTCACCAC AAACGGTAAG ACTTACACCG TCACTGAACC

2651 AACCACTTTG ACTATCACTG ACTGTCCATG TACTATTGAA AAGAGCGAAG

2701 CCCCTGAGTC TTCTGTCCCA GTTACCGAAT CTAAGGGCAC TACCACCAAA

2751 GAAACAGGTG TTACTACCAA ACAAACCACA GCCAACCCAA GTCTAACCGT

2801 CTCCACAGTC GTCCCAGTTT CATCCTCTGC TTCTTCTCAT TCCGTTGTCA

2851 TCAACAGTAA CGGTGCTAAC GTCGTCGTTC CAGGTGCTTT AGGTTTGGCT

← *SED1*

2901 GGTGTTGCTA TGTTATTCTT ATAAACGGTG GTGTTTGACA CATCCGCCTT

2951 CTTAATGCTT TCTTTCAGTA TTATGTTATT TTTTTGTTAT TCGTTTTTCA

3001 CTTCTAGGCT TTTTGACAGA CTAGCCCCGT TATACCACCA TCTTTGTGGG

3051 AAAGCCCCTA AATTGCCCTG AGCAGTATCG TTTCATGTCT AGTCTCTTTA

3101 AAGATGTTTC TTACGCGTTG CGTGTAAAAC ATCCTCTCAT TCAAGACAGG

3151 GTTTTCTAAA AGCAATAGGG GTAGTTTAAT AATTCTTATA TAATCATCAT

3201 ATACACTATT TTTAGTTCTT AATTCTTTAA TACAAACTTA TTAATGTGCT

3251 CTCCATTGAT CTCTTAATCA GGAGGCGATA TATACCGGAA GCGGTGTACT

3301 TTTCTTCACC TCTTACTCAA CTATGTTGAT GTGCAAGTTT AACCACTCGT

3351 CGATATTATC TATTGCTATA ACGAAAACTT TATTCGAGTT CACAGTGAAA

3401 AACTTCAGCA CATTTATGGA AGATCTAAGC AAAATGGAGA ACGCCAGTAG

3451 ATGCGAACAA CAAACTTTAT CAAATTTGAA ATACCACTGC TTTGATAAGC

*Bam*HI

3501 TATGA**GGATC C**AGCTGTGCA AACCTATCAT CCGAAAATTG CAAATGATCA

3551 TCAACTTCAT TTATTAGCAC ATCGTGTAAT TCTTCTTTTT CGTCAGTTTT

3601 TTCTAGTGCT TCATGAAAGT ATTTACAAAA CTCTTCACAT GAGCAGAACC

3651 AGTGTGCGAT ATCTACGAGT ATTGGTGGCG CTCCATCATT AGTATCCTTC

3701 ACTATTAAGC GATACTCCAG GAGCGAATTA GATGAACCCT TATAAAATTC

3751 ATCAACTAAC ACATCAATCA ATGATGTATG CTCATTACCT TCCTTGTTAT

3801 GGACTCTATC TAATATGTAT ATAAACATGT CTGAAGATTC TAGTAATGAA

3851 ATTGCTCGAA T

HA-epitope sequence insert (underlined). From Shimoi *et al.*, 1998.

5'-TCGACTACTTTGGCCTACCCATACGACGTCCCAGACTACGCTCAATTTTCCAACAGT-3'

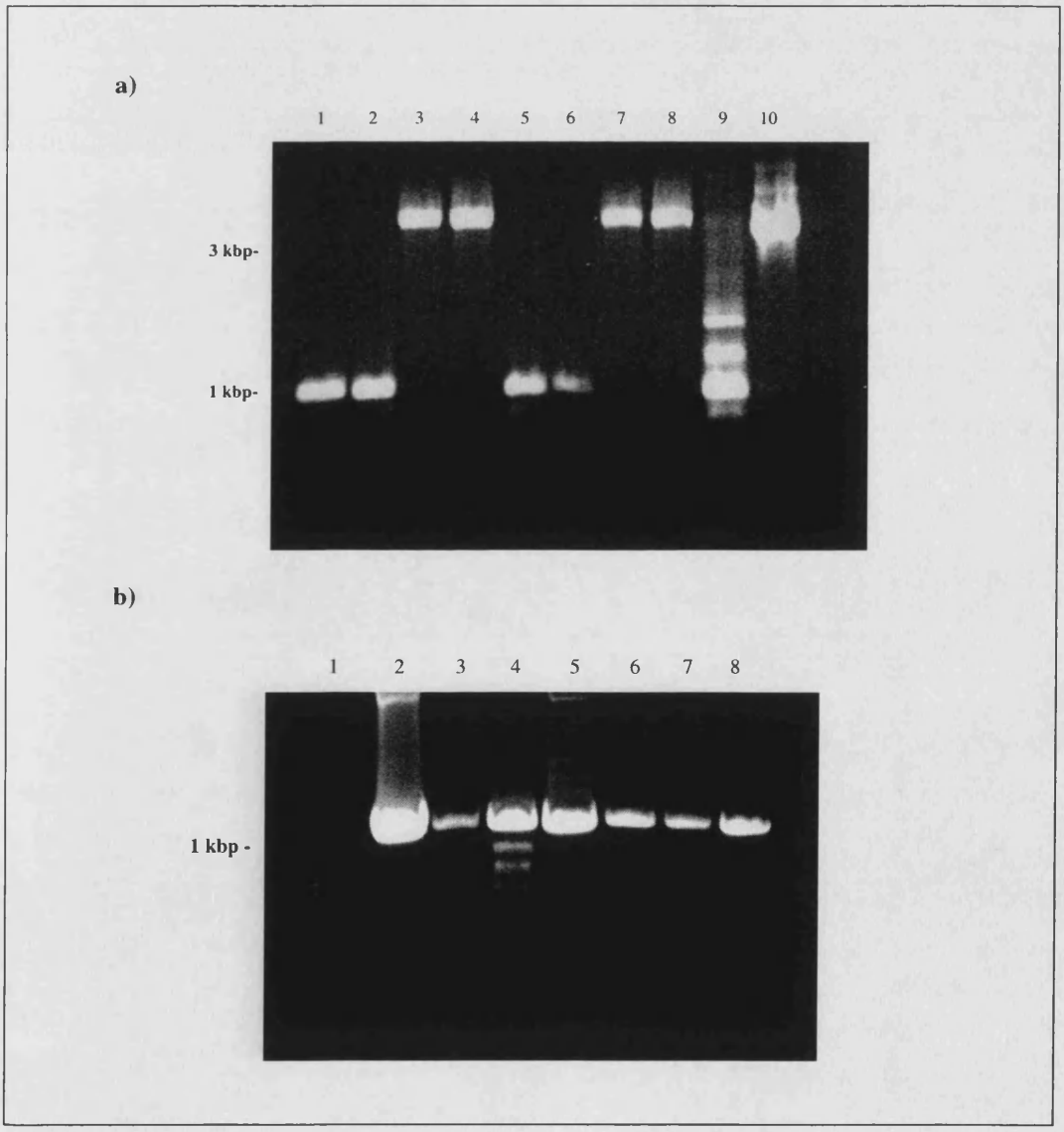
Figure 5.5.4.

a) Synthesis of a *SED1* gene with HA-epitope insert by PCR overlap extension technique.

Lanes 1,2, 5 and 6: *Hin*dIII/*Eco*RI flanked *SED1* with HA epitope insert, constructed from two PCR products. Lanes 3, 4, 7 and 8: *Bam*HI/*Eco*RI flanked *SED1* fragment with HA epitope insert, constructed from two PCR products. Lane 9: *Hin*dIII/*Eco*RI flanked *SED1* control with no epitope tag, PCR product of gDNA from AR27. Lane 10: *Bam*HI/*Eco*RI flanked *SED1* fragment control with no epitope tag, PCR product of gDNA from AR27.

b) PCR product of whole cells transformed with pRS416-HA::*SED1*. One primer consisted of HA-epitope tag sequence. Lane 1: Untransformed AR27. Lane 2: pRS416-HA::*SED1*. Lanes 3-8: AR27, *cwh13, cwh30, cwh41, cwh47, cwh48.*

Electrophoresis was performed on 1 % agarose gels.



Although the PCR technique was successfully completed there was difficulty inserting the PCR product into a suitable vector.

This study used plasmid pRS416-HA::*SED1* which contains an HA 12CA5 epitope tagged *SED1* gene created by site directed mutagenesis (Shimoi *et al.*, 1998). The epitope tagged *SED1* gene resides in a 3.3kb *Eco*RI/*Bam*HI digested fragment which contains the full *SED1* promoter region (Fig. 5.5.3).

AR27 *sed1Δ* and the *kre sed1Δ* strains were successfully transformed with pRS416-HA::*SED1*. This was confirmed by performing PCR analysis on transformed cells using an oligonucleotide of the HA-epitope sequence as a primer (Fig. 5.5.4). The transformed *kre* and parental strains were grown for two weeks in selective SC medium without uracil. Cell walls were then isolated and CWPs were extracted by laminarinase. SDS-PAGE analysis revealed a smeared protein that was approximately 300-350 kDa in size after treatment with laminarinase (Fig 5.5.5). Therefore, glycosylation of the protein seemed to be unaffected by the nature of the mutation in each strain. This study also discovered that all the *kre* strains incorporate a reduced amount of Sed1p in the cell walls (Fig. 5.5.5).

To confirm that the *kre* strains did not contain Sed1p that could be extracted by SDS, western blot analysis was also performed on SDS-extracted proteins. In this case no epitope signal was detected. In *cwh48/kre6* the detection method used could not locate Sed1p in the cell wall but it was the only strain which revealed Sed1p in the intracellular extract (Fig. 5.5.5). This confirmed previous observations in blot tests, where intracellular and cell wall extracts were spotted

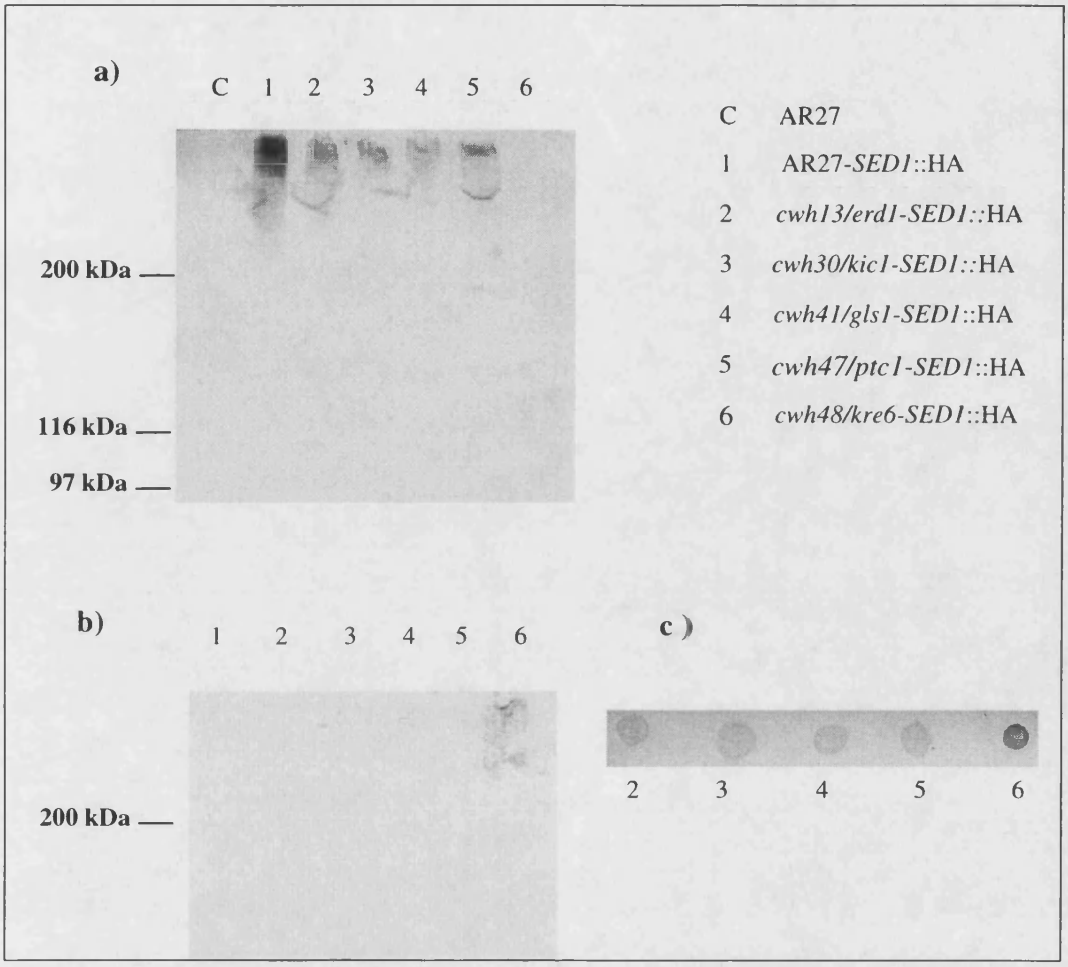


Figure 5.5.5: Western blot analysis of HA-tagged Sed1p. Cells were cultured with shaking for 14 days at 30°C. Proteins were extracted from cell walls with laminarinase (**a**) or isolated from cytoplasm by cell disruption followed by centrifugation (**b+ c**). They were then either applied to SDS-PAGE (3-15% gradient gel) and transferred to a PVDF membrane (**a+b**) or transferred directly to a membrane. The membrane was probed for tagged protein with anti-HA-peroxidase conjugated monoclonal antibodies.

directly onto PVDF membrane and then treated with antibodies directed towards the HA tag (Fig. 5.5.5). Sed1p is not successfully incorporated into the walls of strains with a mutation in the *KRE6* gene.

The *KRE6* gene product is a type II membrane protein which is required for the synthesis of β-glucan (Roemer and Bussey, 1991). Mutations have a reduction in both β1,3-glucan and β1,6-glucan, enlarged cells and a decreased level of alkali-insoluble cell wall proteins (Roemer and Bussey, 1991). Mutants also have reduced glucan synthase activity. This can be corrected by transforming strains with *KRE6* but is reduced if *KRE6* is over-expressed (Roemer and Bussey, 1991).

Mutations in the *KRE6* gene results in a 50% loss of β1,6-glucan in the cell wall (Ram *et al.*,1995). The localisation of Kre6p in the Golgi complex indicates that it is synthesised within the secretory pathway (Roemer *et al.*, 1994). Several studies involving killer resistant strains have revealed that β1,6-glucan is incorporated into glucanase extractable proteins via the secretory pathway (Boone *et al.*, 1990; Jiang *et al.*, 1996; Montijn *et al.*, 1995). GPI-proteins that are similar to Sed1p such as α-agglutinin, Cwp1p and Tip1p are thought to possess a β1,6-glucan side chain that is linked to the GPI-anchor prior to its transglycosylation into the cell wall (Montijn *et al.*, 1995). The incorporation of these proteins into the cell wall is reduced when strains have a mutation that affects the synthesis of β1,6-glucan. Western analysis using affinity purified β1,6-glucan antibodies revealed that proteins incorporated into the cell wall of a wild type strain could not be detected in a strains with a deletion in *KRE6* and

SKN1 (Montijn *et al.*, 1995). As in *KRE6*, the *SKN1* gene also encodes a type II membrane protein which is located in the Golgi complex and is *K1* killer resistant. Deletion of both genes results in a 90% reduction in β1,6-glucan levels (Roemer *et al.*, 1994). Montijn *et al.* (1995) have proposed that Kre6p and Skn1p are glycosyl transferases because they are involved in the modification of N-chains and in the modification of GPI-anchors. This theory is supported by research in another study which demonstrated that glycan side-chains of GPI - anchors are further processed by novel yet unidentified α1,3- and α1,2- mannosyl transferases located in the Golgi complex (Sipos *et al.*, 1995).

5.6 Summary

The PCR product of *SED1* revealed polymorphism in the gene between wine producing strains and laboratory strains. This could be attributed to recombination between brewing strains as a consequence of a more heterogeneous environment. Further investigations could involve sequencing the PCR products or restriction analysis from different strains to determine the cause of variation in gene size.

Sed1p is a stress-induced protein that is localised in the cell wall. Successful incorporation of Sed1p into the cell wall indirectly involves Kre6p. Of all the *kre* strains transformed with the HA-tagged Sed1p, only one, *cwh48/kre6*, did not incorporate the protein into the cell wall. Cells with a mutation in *KRE6* and a disruption in the *SED1* gene also have a reduced growth rate, increased flocculation and greater sensitivity to Zymolyase during the early stages of stationary phase. Increased Zymolyase sensitivity could be due to a decreased

growth rate. Sed1p is known to suppress the *ERD2* phenotype, a gene that is involved in the retention of ER proteins. Kre6p is also localised within the secretory pathway and in a similar way to the *ERD2* phenotype secretes proteins into the media. Sed1p therefore may have a stabilising effect within the secretory pathway and could be indirectly involved in retaining proteins. The other *kre* strains, *cwh13/erd1, cwh30/kic1, cwh41/gls1* and *cwh47/ptc1,* all incorporated Sed1p into the cell wall although at a reduced rate when compared to the parental strain AR27.

Summary

When yeast cells are challenged with nutrient limitation or other extreme environments they adapt by altering their metabolic and physical state. These changes include cessation of cell division, accumulation of storage carbohydrates, condensation of chromosomal DNA and modification of the cell wall. Collectively this stress resistant state is known as stationary phase. Mutations that affect the viability of yeast cells in stationary phase can occur in a number of pathways regulating cellular processes. This work concentrates on the influence of cell wall structure on cell viability in stationary phase.

A library of strains sensitive to Calcofluor White (*cwh*) and defective in cell wall assembly were isolated by Ram *et al.* (1994). The *cwh* strains had previously been classified into 53 complementation groups and categorised into subgroups according to cell wall mannose/glucose ratio. The *cwh* strains were assessed in this study for loss of viability in stationary phase in different environments. Cell wall composition and phenotype were found to influence the viability of cells according to environmental conditions.

Five of the strains were also *K1* killer toxin resistant (*kre*). *Kre* resistant cells have reduced β1,6-glucan content. Certain stress-related proteins in cell walls are thought to be covalently linked to β1,6-glucan. Part of this work analyses the effects of reduced β1,6-glucan on stress and stationary phase characteristics in *kre*. The *kre* strains assessed were found to have variation in stationary phase characteristics, such as accumulation of trehalose, Zymolyase resistance and cell wall protein content.

To further investigate the effect of cell wall assembly in stationary phase a cell wall protein induced by nutrient limitation, Sed1p, was tagged and quantified in *kre* strains. The nature of the cell wall defect was found to influence the incorporation of the cell wall protein into the cell wall.

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Appendix 1

Media and materials.

Synthetic complete (SC) (1 litre):

Component (a)

yeast nitrogen base	6.7 g
adenine	10.0 mg
uracil (omit for SC-URA)	40.0 mg
tyrosine	50.0 mg
agar (for plates)	30.0 mg
distilled water	to 500 ml

Component (b)

arginine	200 mg
histidine (omit for SC-HIS)	200 mg
isoleucine	600 mg
leucine	600 mg
lysine	400 mg
methionine	100 mg
phenylalanine	600 mg
threonine	1000 mg
tryptophan	400 mg
distilled water	to 100 ml

To prepare, add to component (**a**) the following; 435 ml of sterile water; 10 ml of component (**b**); 50 ml of sterile 40% glucose solution; 5 ml of 1 M Na_2HPO_4 (pH7).

SCGAL-URA:

As for synthetic complete medium (SC) except that 50 ml of 40% galactose is substituted for glucose and the uracil is omitted.

YPD (2% (w/w) glucose):

Bacto-yeast extract (1%)	10g
Bacto-peptone (2%)	20g
Glucose (2%)	20g
Agar (2%) (for plates)	20g
Distilled water	930 ml

Selection Plates

Calcofluor-White plates

Stock solution:

Calcofluor-White	1 g
Distilled water	10 ml

Dissolve Calcofluor-White in distilled water filter sterilize (0.2-mm filter) and then add to cooled autoclaved media. Store stock solution at -20°C. To either YPD or synthetic media. Add 5 ml stock solution to 500 ml of autoclaved media (produces [Calcofluor-White] at 1mg/ml).

Copper sulphate plates

1M stock solution:

Copper sulphate	3.46 g
Distilled water	50 ml

Dissolve copper sulphate in distilled water, filter sterilize (0.2-μm filter) and then add to autoclaved medium (~40°C). Add copper sulphate in the required quantity eg. 1.5 ml gives 1.5 mM [copper sulphate].

SDS-PAGE gradient gel.

Gradient gel (3.75-14.8%)	**3.75%**	**14.8%**
Component 1		
Acryl(40%) / Bis (3%)	0.225 ml	0.9 ml
Tris-HCl (8.8 pH)	0.6 ml	0.6 ml
Glycerol	—	0.9 ml
dH_2O	1.53 ml	—
SDS (10%)	26 µl	26 µl
Component 2		
TEMED	5 µl	2.5 µl
APS (10%)	15 µl	7.5 µl

Add the first column of component 1 to the half of a linear gradient maker closest to an attached peristaltic pump. Add the second column of component 1 to the other half of the linear gradient maker. While gently stirring add the second component to the appropriate half of the linear gradient maker, TEMED first then freshly prepared cross-linking agent APS (10%). Immediately after adding the APS (10%) open the tap that divides the two solutions and allow the peristaltic pump to transfer the gel into an assembled casting unit. Insert comb and leave to polymerise for approximately 2 hours.

PCR solutions.

***SED1* amplification.**

5 µl (x10) NH_4 buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, 0.1% Tween-20), 5 µl 0.2 mM dNTPs, 3 µl 50 mM $MgCl_2$, 1 µM Primers, 0.5 µg DNA template, 0.5 µl 25U DNA Taq polymerase (Bioline) in a final volume of 50 µl with deionised dH_2O and overlaid with light mineral oil (50µl).

***KanMX* amplification.**

5 µl (x10) NH_4 buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, 0.1% Tween-20), 5 µl 0.2mM dNTPs, 3 µl 50 mM $MgCl_2$, 1 µM Primers, 0.5 µg DNA template, 0.5 µl 25U DNA Bio-X-act polymerase (Bioline). Made up to 50 µl with deionised dH_2O and overlaid with light mineral oil (50 µl).

***E.coli* cells.**

5 µl (x10) NH_4 buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, 0.1% Tween-20), 0.2mM dNTPs, 3 µl 50 mM $MgCl_2$, 1 µM Primers, 0.5 µl 25U DNA Taq polymerase (Bioline). Made up to 50 µl with deionised dH_2O and overlaid with light mineral oil (50µl).

Overlap extension PCR hybridisation.

Amplification of fragments with overlaps.

5 µl (x10) NH_4 buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, 0.1% Tween-20), 0.2mM dNTPs, 3 µl 50 mM $MgCl_2$, 1 µM Primers, 0.5 µg DNA template, 0.5 µl 25U DNA Taq polymerase (Bioline). Made up to 50 µl with deionised dH_2O and overlaid with light mineral oil (50µl).

Amplification of gene with inserted epitope tag.

5 µl (x10) Expand buffer (with $MgCl_2$), 0.2 mM dNTPs, 1 µM Primers, 0.5 µg DNA template, 0.7 µl Expand Polymerase Taq (Boehringer Mannheim). Made up to 50 µl with de-ionised dH_2O and overlaid with light mineral oil (50 µl).

Appendix 2

Screening data.

Statistical Information- Yeast groups.		Phloxine B viability stain.								
		Phloxine B Control (28°C)				1M sorbitol				
		Exp	1wk	3wk	5wk	Exp	1wk	3wk	5wk	Strain ID
Parental strains:		3	1	1	0	3	2	3	3	AR49
		3	2	2	2	3	2	3	0	AR48
		3	2	3	2	3	2	3	0	AR32
		3	2	2	2	3	2	3	2	AR31
		3	1	2	2	3	1	3	0	AR29
		3	1	1	2	2	1	2	2	AR27
	Total:	18	9	11	10	17	10	17	7	Total:
	Mean:	3.00	1.50	1.83	1.67	2.83	1.67	2.83	1.17	Mean:
	SEM:	0.00	0.22	0.31	0.33	0.17	0.21	0.17	0.54	SEM:
Group 3:		3	1	1	2	3	1	2	2	53-1a
		1	1	2	2	1	1	2	3	53-1α
		1	1	1	1	1	1	2	2	52-1a
		1	2	1	3	3	1	2	2	52-1α
		2	1	2	2	1	1	1	1	51a
		1	1	3	3	1	1	2	1	50α
		3	1	1	2	3	1	1	3	50a
		3	1	2	2	3	1	1	1	49a
		3	1	2	2	3	1	2	2	49α
		1	1	2	2	3	1	2	2	48a
		3	1	2	2	3	1	2	2	48α
		1	1	1	1	3	1	1	2	47a
		2	2	2	3	3	1	1	2	47α
		1	1	1	1	1	1	3	3	46-2a
		3	1	1	1	2	1	2	3	46-2α
		1	1	1	1	2	1	2	2	46-1a
		1	1	1	2	1	1	2	3	46-1α
		3	2	2	2	2	2	3	2	45α
		3	2	2	2	2	2	2	2	45a
		3	2	2	2	3	2	2	3	44-2a
		3	1	1	1	3	1	2	3	44-2α
		3	1	1	2	3	1	2	2	44-1a
		3	2	2	2	3	2	2	2	43-2a
		3	2	2	2	3	2	2	2	43-1α
		3	2	3	2	3	2	3	2	42α
		3	2	2	1	3	2	1	2	41α
		2	1	1	1	3	1	1	2	41a
	Total:	60	36	44	49	65	34	50	58	Total:
	Mean:	2.22	1.33	1.63	1.81	2.41	1.26	1.85	2.15	Mean:
	SEM:	0.18	0.09	0.12	0.12	0.16	0.09	0.12	0.12	SEM:
	n	27	27	27	27	27	27	27	27	*n*
Group 2:		3	2	2	2	3	2	1	1	37a
		3	2	2	2	1	2	2	2	33α
		3	2	2	2	3	2	2	2	40α
		3	1	1	1	3	1	2	2	39a
		3	1	1	1	3	1	1	2	33a
		3	2	1	3	3	2	2	2	34α
		1	2	3	2	3	2	3	0	36a
		1	2	3	2	3	2	3	2	36α
		1	2	2	3	3	2	3	2	32a
		3	2	2	3	3	2	3	3	29a
		3	2	2	2	2	2	2	3	29α
		3	2	2	3	3	2	2	3	28α
		3	nd	nd	nd	3	2	1	3	27a
		1	nd	nd	nd	3	2	2	2	26α
		1	1	1	1	3	1	2	2	25a
		1	1	1	1	3	1	2	2	25α
		3	1	2	0	3	1	2	2	23a
	Total:	39	25	27	28	48	29	35	35	Total:

	Mean:	2.29	1.67	1.80	1.87	2.82	1.71	2.06	2.06	Mean:
	SEM:	0.24	0.15	0.19	0.24	0.13	0.11	0.16	0.18	SEM:
	n	17	15	15	15	17	17	17	17	*n*
Group 1:		3	2	1	1	3	2	2	2	22a
		3	2	1	1	3	2	2	3	22α
		3	2	2	2	3	2	2	2	21α
		3	2	2	2	1	2	3	3	20a
		2	1	1	2	3	2	2	2	11α
		3	2	2	2	3	2	2	2	10α
		3	1	1	1	2	1	1	2	19a
		3	1	1	1	2	2	2	3	19α
		3	nd	nd	nd	2	2	2	2	18a
		1	2	2	2	1	2	2	2	18α
		2	1	1	1	3	1	3	3	17a
		2	2	3	2	2	2	2	2	17α
		2	1	1	1	3	2	2	3	16a
		1	2	2	2	3	2	3	2	15α
		1	1	2	3	3	2	3	3	14α
		2	2	2	2	3	1	2	2	13a
		2	2	2	3	3	2	2	2	13α
		2	2	2	3	3	2	3	3	12a
		2	2	2	3	3	2	3	2	12α
		1	2	0	0	3	0	0	0	11a
		1	1	1	1	2	1	2	1	9a
		1	2	1	1	2	2	2	1	8a
		2	2	2	2	3	2	2	3	8α
		1	0	0	0	3	2	3	3	7α
		1	2	2	3	3	2	3	3	6a
		1	2	1	3	3	2	3	2	5a
		1	1	1	2	2	2	3	2	5α
		1	1	1	3	1	2	2	2	4a
		1	2	2	3	1	2	2	1	4α
		1	2	2	1	1	2	2	1	3a
		2	2	2	2	3	2	3	3	3α
		2	2	1	1	3	2	2	3	2a
		1	2	2	2	1	2	2	2	2α
		1	2	2	3	1	2	2	3	1α
	Total:	61	55	50	61	81	62	76	75	Total:
	Mean:	1.79	1.67	1.52	1.85	2.38	1.82	2.24	2.21	Mean:
	SEM:	0.14	0.10	0.12	0.16	0.14	0.08	0.11	0.13	SEM:
	n	34	33	33	33	34	34	34	34	*n*

Red font indicates a strain were the defective gene has been isolated.

Blue font indicates wild type strain.

** Phloxine B stain scored 0 - 3 (no growth - high viability).

Yeast cell were grown for the specified time in YPD containing 0.3% glucose, +/- sorbitol.

Cultures were then spotted on to SC media containing 2% (w/v) glucose + 1mg/ml Phloxine B.

nd, not determined.

Strain ID	Exp phase. Phloxine B			1 wk (stationary phase) Phloxine B			3 wks (stationary phase) Phloxine B			5 wks (stationary phase) Phloxine B		
	14°C	37°C	28°C	14°C	37°C	28°C	14°C	37°C	28°C	14°C	37°C	28°C
AR49	3	1	3	3	2	1	3	3	1	3	3	0
AR48	3	1	3	3	3	2	3	3	2	3	3	2
AR32	3	1	3	3	3	2	3	3	3	3	3	2
AR31	3	1	3	3	2	2	0	2	2	2	2	2
AR29	3	1	3	2	2	1	3	3	2	2	2	2
AR27	3	1	3	2	2	1	1	2	1	1	0	2
Total:	18	6	18	16	14	9	13	16	11	14	13	10
Mean:	3	1	3	2.67	2.33	1.50	2.17	2.67	1.83	2.33	2.17	1.67
SEM:	0	0	0	0.21	0.21	0.22	0.54	0.21	0.31	0.33	0.48	0.33
53-1a	3	1	3	2	1	1	0	2	1	2	2	2
53-1α	3	1	0	0	0	1	0	3	2	2	2	2
52-1a	3	1	0	3	2	1	2	2	1	2	2	1
52-1α	3	1	0	2	2	2	2	2	1	2	2	3
51a	3	1	2	0	2	1	1	1	2	2	2	2
50α	1	1	0	0	1	1	0	1	3	0	2	3
50a	3	1	3	3	1	1	2	2	1	2	1	2
49a	3	1	3	3	2	1	2	2	2	1	1	2
49α	2	1	2	3	2	1	2	2	2	2	2	2
48a	3	1	0	3	2	1	3	2	2	2	2	2
48α	3	1	3	3	2	1	2	2	2	2	2	2
47a	3	1	0	0	1	1	1	1	1	0	2	1
47α	3	1	2	2	1	2	1	2	2	0	1	3
46-2a	3	1	0	2	2	1	1	2	1	0	1	1
46-2α	3	1	3	2	2	1	0	2	1	2	2	1
46-1a	1	1	0	3	2	1	2	2	1	2	2	1
46-1α	3	1	0	3	2	1	2	2	1	2	2	2
45α	3	1	3	3	2	2	2	2	2	2	2	2
45a	3	1	3	2	2	2	2	2	2	2	2	2
44-2a	3	1	3	2	2	2	2	2	2	2	2	2
44-2α	3	1	3	2	2	1	2	2	1	2	2	1
44-1a	3	1	3	2	2	1	2	2	1	2	2	2
43-2a	3	1	3	2	2	2	2	2	2	0	2	2
43-1α	3	1	3	3	2	2	2	2	2	2	2	2
42α	3	1	3	3	2	2	3	2	3	1	2	2
41α	3	1	2	3	2	2	2	2	2	2	1	1
41a	2	1	3	2	2	1	2	2	1	2	2	1
Total:	75	27	50	58	47	36	44	52	44	42	49	49
Mean:	2.78	1.00	1.85	2.15	1.74	1.33	1.63	1.93	1.63	1.56	1.81	1.81
SEM:	0.11	0.00	0.27	0.20	0.10	0.09	0.16	0.07	0.12	0.15	0.08	0.12
n	27	27	27	27	27	27	27	27	27	27	27	27
37a	3	1	3	2	1	2	0	1	2	0	1	2
33α	3	1	3	3	3	2	3	3	2	2	2	2
40α	3	1	3	2	2	2	0	2	2	2	2	2
39a	3	1	3	0	2	1	1	2	1	2	1	1
33a	3	1	3	3	2	1	2	2	1	2	2	1
34α	2	1	3	2	2	2	2	2	1	2	2	3
36a	1	1	0	3	2	2	0	0	3	2	2	2
36α	1	1	0	0	0	2	3	3	3	2	2	2
32a	1	1	0	0	0	2	0	0	2	2	2	3
29a	3	1	3	3	2	2	3	2	2	2	2	3
29α	3	1	3	3	2	2	2	2	2	2	2	2
28α	3	1	3	3	0	2	3	0	2	2	2	3
27a	3	1	3	3	2	1	3	2	1	2	2	1
26α	1	1	0	3	2	0	2	2	0	2	2	0
25a	2	1	1	2	1	1	2	1	1	1	2	1
25α	2	1	1	2	1	1	0	1	1	1	2	1
23a	3	1	3	3	2	1	3	2	2	0	2	0
Total:	40	17	35	37	26	26	29	27	28	28	32	29
Mean:	2.35	1.00	2.06	2.18	1.53	1.53	1.71	1.59	1.65	1.65	1.88	1.71
SEM:	0.21	0.00	0.33	0.27	0.21	0.15	0.31	0.23	0.19	0.17	0.08	0.24
n	17	17	17	17	17	17	17	17	17	17	17	17
22a	3	1	3	2	1	2	3	1	1	2	3	1
22α	3	1	3	3	2	2	2	2	1	2	3	1

21α	3	1	3	3	2	2	2	2	2	1	2	2
20a	3	1	3	2	2	2	1	2	2	2	1	2
11α	3	1	2	3	2	1	3	2	1	2	3	2
10α	3	1	3	2	2	2	3	2	2	1	2	2
19a	3	1	3	2	0	1	3	0	1	1	2	1
19α	3	1	3	0	1	1	2	1	1	2	2	1
18a	3	1	3	2	3	0	3	3	0	2	2	0
18α	1	1	0	2	2	2	2	2	2	2	2	2
17a	2	1	2	3	0	1	2	0	1	2	1	1
17α	2	1	2	3	3	2	2	3	3	2	2	2
16a	2	1	2	3	1	1	2	1	1	2	1	1
15α	2	1	3	2	1	2	0	1	2	1	1	2
14α	3	1	0	2	2	1	2	2	2	3	2	3
13a	2	1	2	2	2	2	2	2	2	2	2	2
13α	2	1	2	2	3	2	2	3	2	2	2	3
12a	3	1	2	3	2	2	2	2	2	2	1	3
12α	3	1	2	2	2	2	3	2	2	2	2	3
11a	2	1	1	3	2	2	3	2	0	2	2	0
9a	1	1	1	3	2	1	3	2	1	1	1	1
8a	1	1	0	3	3	2	2	2	1	2	2	1
8α	2	1	2	2	2	2	2	2	2	1	2	2
7α	1	1	0	0	0	0	0	0	0	0	0	0
6a	1	1	0	2	2	2	3	2	2	2	2	3
5a	2	1	2	2	2	2	2	2	1	1	2	1
5α	2	1	1	2	2	1	2	1	1	1	2	2
4a	1	1	0	2	0	1	3	1	1	1	1	3
4α	1	1	0	0	0	2	0	2	2	2	1	3
3a	2	1	1	3	2	2	3	2	2	2	2	1
3α	2	1	2	0	1	2	2	1	2	1	1	2
2a	2	1	2	3	1	2	3	1	1	1	2	1
2α	2	1	1	2	1	2	3	1	2	2	2	2
1α	1	1	0	2	0	2	2	1	2	1	1	3
Total:	72	34	56	72	53	55	74	55	50	55	59	59
Mean:	2.12	1.00	1.65	2.12	1.56	1.62	2.18	1.62	1.47	1.62	1.74	1.74
SEM:	0.13	0.00	0.19	0.16	0.16	0.10	0.15	0.13	0.12	0.10	0.11	0.16
n	34	34	34	34	34	34	34	34	34	34	34	34

Red font indicates a strain were the defective gene has been isolated.

Blue font indicates wild type strain.

** Phloxine B stain scored 0 - 3 (no growth - high viability).

Yeast cell were grown for the specified time in YPD containing 0.3% glucose, +/- sorbitol.

Cultures were then spotted on to SC media containing 2% (w/v) glucose + 1mg/ml Phloxine B.

nd, not determined.

1 wk + 1M sorbitol. Phloxine B			3 wks + 1M sorbitol Phloxine B			5 wks + 1M Sorbitol Phloxine B					
14°C	37°C	28°C	14°C	37°C	28°C	14°C	37°C	28°C	group	α = 1 a = 0	Strain ID
3	2	2	2	2	3	2	2	3			AR49
3	3	2	2	2	3	2	2	0			AR48
3	3	2	3	3	3	3	2	0			AR32
3	2	2	2	1	3	2	2	2			AR31
3	3	1	3	3	3	3	3	0			AR29
3	2	1	0	1	2	1	1	2			AR27
18	15	10	12	12	17	13	12	7			Total:
3.00	2.50	1.67	2.00	2.00	2.83	2.17	2.00	1.17			Mean:
0.00	0.22	0.21	0.45	0.37	0.17	0.31	0.26	0.54			SEM:
2	2	1	0	1	2	2	2	2	3	1	53-1a
2	2	1	0	1	2	2	2	3	3	1	53-1α
2	2	1	0	1	2	2	2	2	3	1	52-1a
2	2	1	2	1	2	2	2	2	3	0	52-1α
2	1	1	2	1	1	2	1	1	3	1	51a
3	1	1	2	1	2	0	2	1	3	1	50α
2	1	1	2	1	1	2	2	3	3	1	50a
2	1	1	2	1	1	1	1	1	3	0	49a
2	2	1	3	1	2	1	1	2	3	1	49α
2	2	1	3	1	2	2	1	2	3	0	48a
3	2	1	3	1	2	2	1	2	3	0	48α
2	2	1	3	1	1	1	2	2	3	1	47a
2	2	1	1	1	1	2	2	2	3	1	47α
0	2	1	1	1	3	2	2	3	3	0	46-2a
2	2	1	2	1	2	2	2	3	3	1	46-2α
2	2	1	2	1	2	2	2	2	3	0	46-1a
0	0	1	2	1	2	2	2	3	3	0	46-1α
2	2	2	3	2	3	2	2	2	3	1	45α
2	2	2	2	1	2	2	2	2	3	0	45a
2	2	2	2	1	2	2	1	3	3	0	44-2a
3	2	1	3	1	2	2	2	3	3	1	44-2α
3	2	1	2	1	2	1	2	2	3	0	44-1a
3	2	2	0	1	2	1	2	2	3	1	43-2a
3	2	2	2	1	2	0	2	2	3	1	43-1α
2	1	2	2	1	3	2	2	2	3	1	42α
3	2	2	2	1	1	2	1	2	3	1	41α
2	1	1	2	1	1	2	1	2	3	0	41a
57	46	34	50	28	50	45	46	58			Total:
2.11	1.70	1.26	1.85	1.04	1.85	1.67	1.70	2.15			Mean:
0.14	0.10	0.09	0.18	0.04	0.12	0.12	0.09	0.12			SEM:
27	27	27	27	27	27	27	27	27			*n*
2	1	2	2	1	1	2	2	1	2	0	37a
3	2	2	3	2	2	2	2	2	2	0	33α
2	2	2	3	2	2	2	2	2	2	1	40α
2	2	3	2	2	2	3	2	2	2	0	39a
3	2	3	2	1	1	1	1	2	2	0	33a
2	2	2	1	1	2	2	2	2	2	0	34α
0	0	2	0	0	3	0	2	0	2	1	36a
2	2	2	0	0	3	2	2	2	2	0	36α
2	2	2	1	1	3	2	1	2	2	0	32a
0	2	2	0	0	3	2	2	3	2	0	29a
2	2	2	1	1	2	2	1	3	2	0	29α
3	2	2	0	2	2	2	2	3	2	1	28α
3	2	2	3	2	1	1	2	3	2	1	27a
0	0	2	2	2	2	1	2	2	2	0	26α
1	1	1	1	1	2	1	2	2	2	0	25a
1	1	1	1	1	2	1	2	2	2	1	25α
3	3	1	1	1	2	2	2	2	2	0	23a
31	28	33	23	20	35	28	31	35			Total:
1.82	1.65	1.94	1.35	1.18	2.06	1.65	1.82	2.06			Mean:
0.26	0.19	0.13	0.26	0.18	0.16	0.17	0.10	0.18			SEM:
17	17	17	17	17	17	17	17	17			*n*
2	2	2	3	1	2	2	2	2	1	0	22a
2	2	2	3	1	2	2	2	3	1	0	22α

2	2	2	2	1	2	2	1	2	1	1	21α
0	1	2	2	1	1	2	2	3	1	1	20a
3	1	2	3	1	2	2	2	2	1	0	11α
2	2	2	3	1	2	2	2	2	1	1	10α
2	1	1	3	1	1	1	1	2	1	1	19a
0	0	2	3	1	2	2	2	3	1	0	19α
1	1	2	3	1	2	2	2	2	1	0	18a
0	1	2	1	1	2	1	1	2	1	1	18α
3	1	1	2	2	3	0	2	3	1	0	17a
3	3	2	2	2	2	3	2	2	1	0	17α
2	1	2	0	0	2	2	2	3	1	1	16a
2	1	2	0	2	3	2	2	2	1	0	15α
2	0	2	0	0	3	0	0	3	1	0	14α
2	2	1	2	2	2	1	2	2	1	0	13a
2	2	2	2	2	2	1	2	2	1	1	13α
2	1	2	2	1	3	2	2	3	1	0	12a
2	1	2	2	1	3	2	2	2	1	1	12α
3	1	0	2	1	0	2	0	0	1	1	11a
2	2	1	2	0	2	2	2	1	1	0	9a
2	2	2	2	1	2	2	2	1	1	0	8a
2	2	2	2	1	2	2	2	3	1	1	8α
2	2	2	2	1	3	2	2	3	1	0	7α
2	2	2	2	2	3	2	2	3	1	1	6a
2	2	2	2	1	3	2	2	2	1	1	5a
2	2	2	2	1	3	1	1	2	1	1	5α
2	2	2	2	1	2	1	1	2	1	1	4a
0	0	2	1	0	2	2	2	3	1	1	4α
2	2	2	1	1	2	2	2	2	1	0	3a
2	2	2	2	1	3	2	2	3	1	0	3α
3	2	2	2	1	2	1	2	3	1	0	2a
2	1	2	2	1	2	2	2	2	1	1	2α
0	1	2	0	1	2	2	2	3	1	1	1α
62	50	62	64	36	74	58	59	78			Total:
1.82	1.47	1.82	1.88	1.06	2.18	1.71	1.74	2.29			Mean:
0.15	0.12	0.08	0.15	0.09	0.12	0.11	0.10	0.12			SEM:
34	34	34	34	34	34	34	34	34			*n*

Red font indicates a strain were the defective gene has been isolated.
Blue font indicates wild type strain.
** Phloxine B stain scored 0 - 3 (no growth - high viability).
Yeast cell were grown for the specified time in YPD containing 0.3% glucose, +/- sorbitol.
Cultures were then spotted on to SC media containing 2% (w/v) glucose + 1mg/ml Phloxine B.
nd, not determined.

Appendix 3

Genetic maps and sequence data.

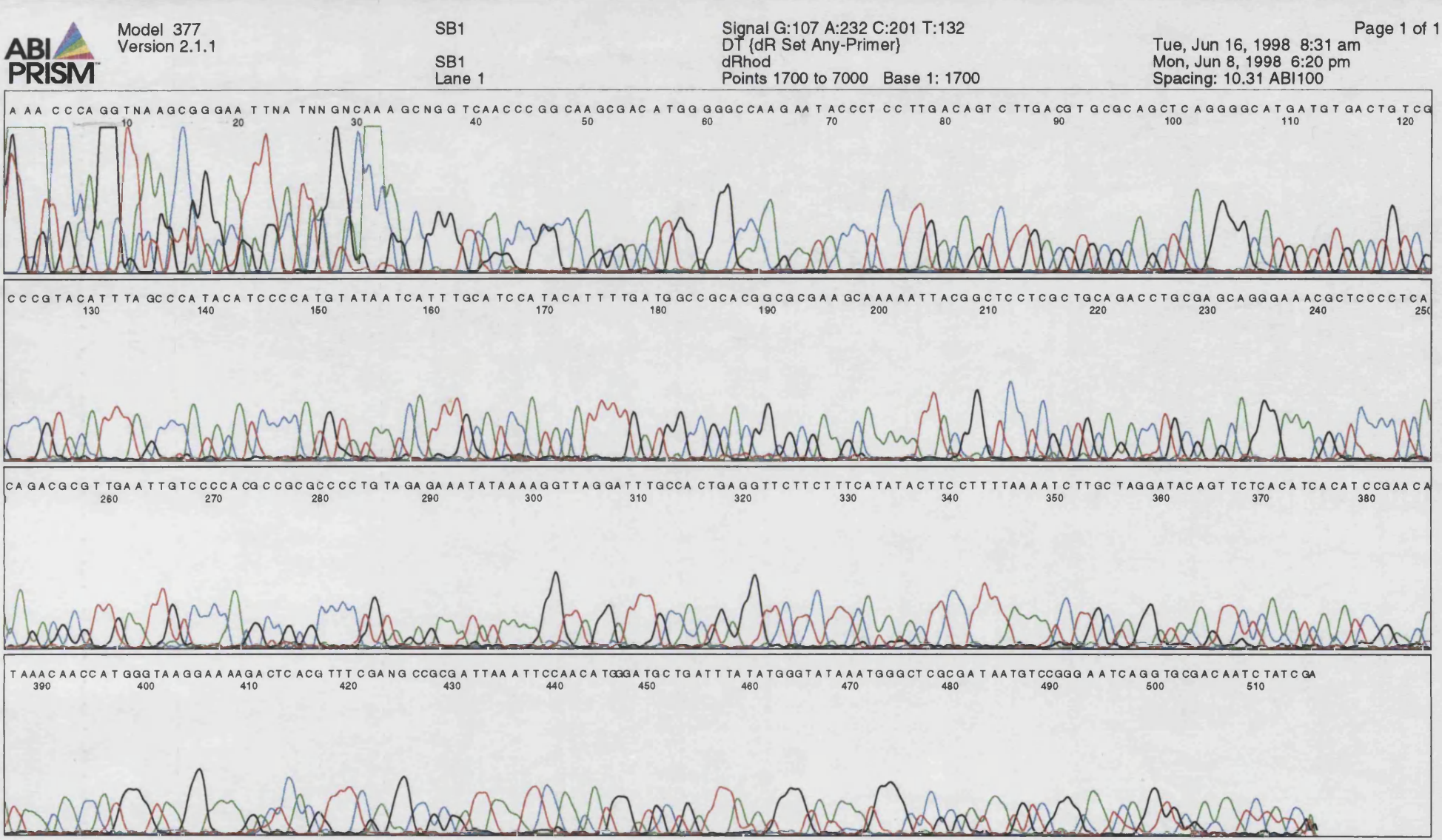
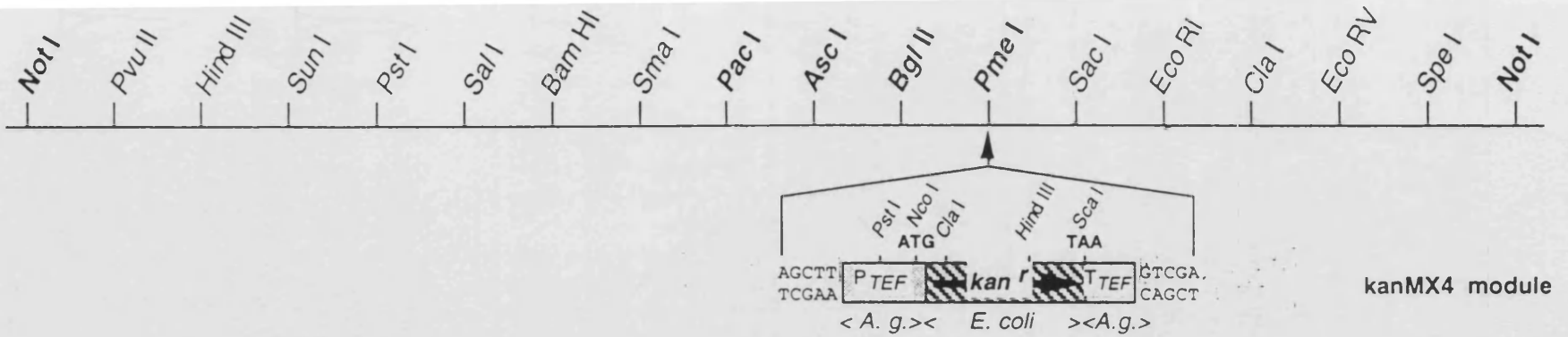


Figure 1a: Chromatogram of DNA sequence obtained from the PCR product of the *Not*I digested plasmid pFA6-*kanMX4*.




```
      EcoR V  Hind III
-386  GATATCAAGCTTGCCTCGTCCCCGCCGGGTCACCCGGCCAGCGACATGGAGGCCCAGAATACCCTCCTTGACAGTCTTGACGTGCGCAGCTCAGGGGCATGATGTGACTGTCGCCCGTAC -267
                                                                                                          Pst I
-266  ATTTAGCCCATACATCCCCATGTATAATCATTTGCATCCATACATTTTGATGGCCGCACGGCGCGAAGCAAAAATTACGGCTCCTCGCTGCAGACCTGCGAGCAGGGAAACGCTCCCCTC -147
           (EcoR I)          (Asc I)
-146  ACAGACGCGTTGAATTGTCCCCACGCCGCGCCCCTGTAGAGAAATATAAAAGGTTAGGATTTGCCACTGAGGTTCTTCTTTCATATACTTCCTTTTAAAATCTTGCTAGGATACAGTTCT  -27
                                Nco I
 -26  CACATCACATCCGAACATAAACAACCATGGGTAAGGAAAAGACTCACGTTTCGAGGCCGCGATTAAATTCCAACATGGATGCTGATTTATATGGGTATAAATGGGCTCGCGATAATGTCG   93
   1                              M  G  K  E  K  T  H  V  S  R  P  R  L  N  S  N  M  D  A  D  L  Y  G  Y  K  W  A  R  D  N  V    31

  94  GGCAATCAGGTGCGACAATCTATCGATTGTATGGGAAGCCCGATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGATGTTACAGATGAGATGGTCAGACTAAACT  213
  32  G  Q  S  G  A  T  I  Y  R  L  Y  G  K  P  D  A  P  E  L  F  L  K  H  G  K  G  S  V  A  N  D  V  T  D  E  M  V  R  L  N    71
                                                                                                  (Sma I)
 214  GGCTGACGGAATTTATGCCTCTTCCGACCATCAAGCATTTTATCCGTACTCCTGATGATGCATGGTTACTCACCACTGCGATCCCCGGCAAAACAGCATTCCAGGTATTAGAAGAATATC  333
 112  W  L  T  E  F  M  P  L  P  T  I  K  H  F  I  R  T  P  D  D  A  W  L  L  T  T  A  I  P  G  K  T  A  F  Q  V  L  E  E  Y   111

 334  CTGATTCAGGTGAAAATATTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCATTCGATTCCTGTTTGTAATTGTCCTTTTAACAGCGATCGCGTATTTCGTCTCGCTCAGGCGCAAT  453
 112  P  D  S  G  E  N  I  V  D  A  L  A  V  F  L  R  R  L  H  S  I  P  V  C  N  C  P  F  N  S  D  R  V  F  R  L  A  Q  A  Q   151

 454  CACGAATGAATAACGGTTTGGTTGATGCGAGTGATTTTGATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATAAGCTTTTGCCATTCTCACCGGATTCAGTCG  573
 152  S  R  M  N  N  G  L  V  D  A  S  D  F  D  D  E  R  N  G  W  P  V  E  Q  V  W  K  E  M  H  K  L  L  P  F  S  P  D  S  V   191

 574  TCACTCATGGTGATTTCTCACTTGATAACCTTATTTTTGACGAGGGGAAATTAATAGGTTGTATTGATGTTGGACGAGTCGGAATCGCAGACCGATACCAGGATCTTGCCATCCTATGGA  693
 192  V  T  H  G  D  F  S  L  D  N  *  I  F  D  *  G  K  L  I  G  C  I  D  V  G  R  V  G  I  A  D  R  Y  Q  D  L  A  I  L  W   231

 694  ACTGCCTCGGTGAGTTTTCTCCTTCATTACAGAAACGGCTTTTTCAAAAATATGGTATTGATAATCCTGATATGAATAAATTGCAGTTTCATTTGATGCTCGATGAGTTTTTCTAATCAG  813
 232  N  C  L  G  E  F  S  P  S  L  Q  K  R  L  F  Q  K  Y  G  I  D  N  P  D  M  N  K  L  Q  F  H  L  M  L  D  E  F  F  *      269
      Sca I
 814  TACTGACAATAAAAAAGATTCTTGTTTTCAAGAACTTGTCATTTGTATAGTTTTTTTATATTGTAGTTGTTCTATTTTAATCAAATGTTAGCGTGATTTATATTTTTTTTCCCTCGACAT  933
                                                                                                          Sal I
 934  CATCTGCCCAGATGCGAAGTTAAGTGCGCAGAAAGTAATATCATGCGTCAATCGTATGTGAATGCTGGTCGCTATACTGCTGTCGATTCGATACTAACGCCGCCATCCAGTGTCGAC  1050
```

Figure 1b: Map and sequence of selector module *kanMX4* (Wach *et al.*, 1994). DNA sequence obtained from PCR product of the *Not*I digested plasmid pFA6-*kanMX4* module aligned with the gene sequence of *kanMX4*. Homologous sequence is over-lined.

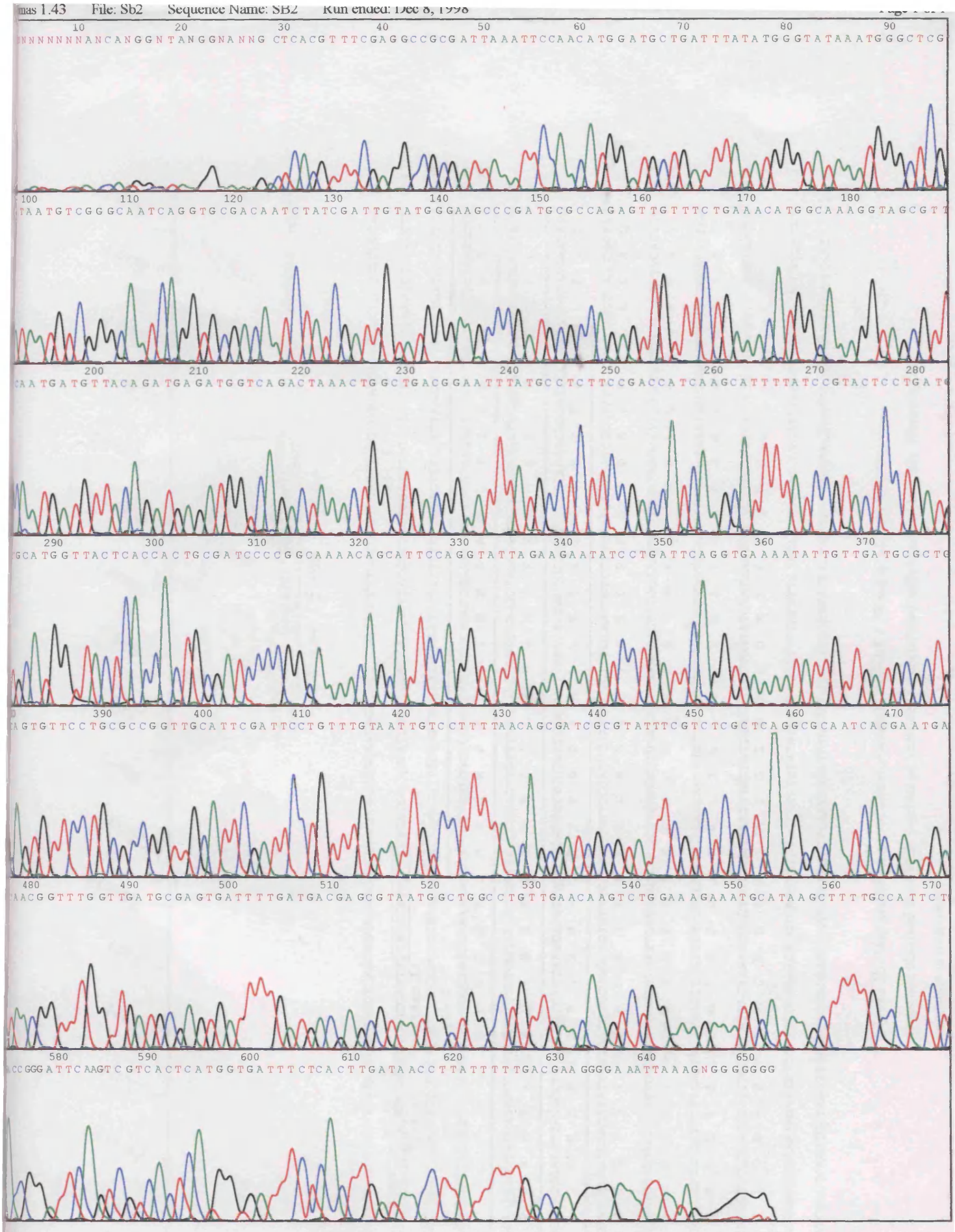
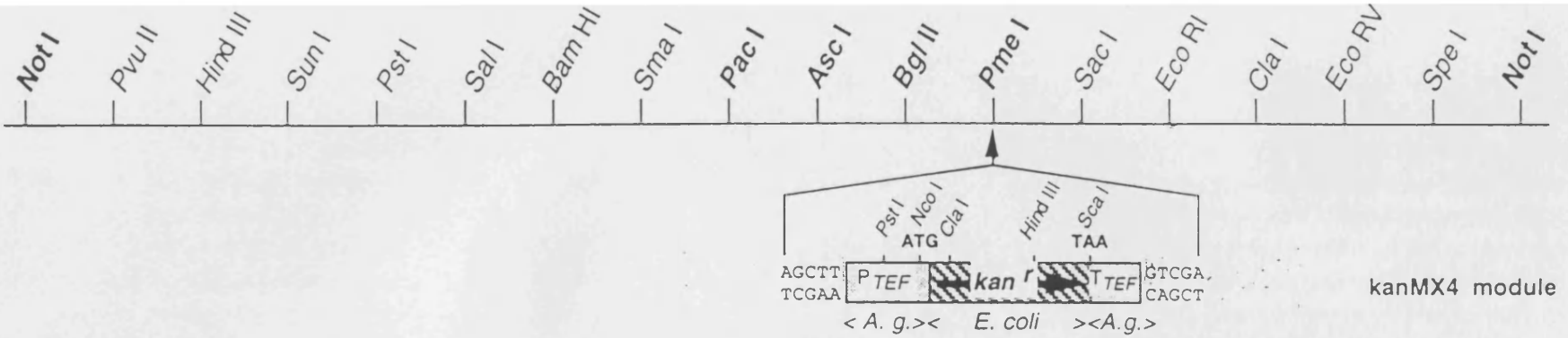


Figure 2a: Chromatogram of DNA sequence obtained from the PCR product of yeast cells transformed with *kanMX4* module.

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     EcoR V  Hind III
-386 GATATCAAGCTTGCCTCGTCCCCGCCGGGTCACCCGGCCAGCGACATGGAGGCCCAGAATACCCTCCTTGACAGTCTTGACGTGCGCAGCTCAGGGGCATGATGTGACTGTCGCCCGTAC -267
                                                                                                          Pst I
-266 ATTTAGCCCATACATCCCCATGTATAATCATTTGCATCCATACATTTTGATGGCCGCACGGCGCGAAGCAAAAATTACGGCTCCTCGCTGCAGACCTGCGAGCAGGGAAACGCTCCCCTC -147
           (EcoR I)          (Asc I)
-146 ACAGACGCGTTGAATTGTCCCCACGCCGCGCCCCTGTAGAGAAATATAAAAGGTTAGGATTTGCCACTGAGGTTCTTCTTTCATATACTTCCTTTTAAAATCTTGCTAGGATACAGTTCT  -27
          Nco I
 -26 CACATCACATCCGAACATAAACAACCATGGGTAAGGAAAAGACTCACGTTTCGAGGCCGCGATTAAATTCCAACATGGATGCTGATTTATATGGGTATAAATGGGCTCGCGATAATGTCG   93
   1                          M  G  K  E  K  T  H  V  S  R  P  R  L  N  S  N  M  D  A  D  L  Y  G  Y  K  W  A  R  D  N  V    31

  94 GGCAATCAGGTGCGACAATCTATCGATTGTATGGGAAGCCCGATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGATGTTACAGATGAGATGGTCAGACTAAACT  213
  32 G  Q  S  G  A  T  I  Y  R  L  Y  G  K  P  D  A  P  E  L  F  L  K  H  G  K  G  S  V  A  N  D  V  T  D  E  M  V  R  L  N    71
                                                                                                  (Sma I)
 214 GGCTGACGGAATTTATGCCTCTTCCGACCATCAAGCATTTTATCCGTACTCCTGATGATGCATGGTTACTCACCACTGCGATCCCCGGCAAAACAGCATTCCAGGTATTAGAAGAATATC  333
  72 W  L  T  E  F  M  P  L  P  T  I  K  H  F  I  R  T  P  D  D  A  W  L  L  T  T  A  I  P  G  K  T  A  F  Q  V  L  E  E  Y   111

 334 CTGATTCAGGTGAAAATATTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCATTCGATTCCTGTTTGTAATTGTCCTTTTAACAGCGATCGCGTATTTCGTCTCGCTCAGGCGCAAT  453
 112 P  D  S  G  E  N  I  V  D  A  L  A  V  F  L  R  R  L  H  S  I  P  V  C  N  C  P  P  N  S  D  R  V  F  R  L  A  Q  A  Q   151

 454 CACGAATGAATAACGGTTTGGTTGATGCGAGTGATTTTGATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATAAGCTTTTGCCATTCTCACCGGATTCAGTCG  573
 152 S  R  M  N  N  G  L  V  D  A  S  D  F  D  D  E  R  N  G  W  P  V  E  Q  V  W  K  E  M  H  K  L  L  P  F  S  P  D  S  V   191

 574 TCACTCATGGTGATTTCTCACTTGATAACCTTATTTTTGACGAGGGGAAATTAATAGCTTGTATTGATGTTGGACGAGTCGGAATCGCAGACCGATACCAGGATCTTGCCATCCTATGGA  693
 192 V  T  H  G  D  F  S  L  D  N  I  I  F  D  E  G  K  L  I  G  C  I  D  V  G  R  V  G  I  A  D  R  Y  Q  D  L  A  I  L  W   231

 694 ACTGCCTCGGTGAGTTTTCTCCTTCATTACAGAAACGGCTTTTTCAAAAATATGGTATTGATAATCCTGATATGAATAAATTGCAGTTTCATTTGATGCTCGATGAGTTTTTCTAATCAG  813
 232 N  C  L  G  E  F  S  P  S  L  Q  K  R  L  F  Q  K  Y  G  I  D  N  P  D  M  N  K  L  Q  F  H  L  M  L  D  E  F  F  *      269
 Sca I
 814 TACTGACAATAAAAAGATTCTTGTTTTCAAGAACTTGTCATTTGTATAGTTTTTTTATATTGTAGTTGTTCTATTTTAATCAAATGTTAGCGTGATTTATATTTTTTTTCGCCTCGACAT  933
                                                                                                          Sal I
 934 CATCTGCCCAGATGCGAAGTTAAGTGCGCAGAAAGTAATATCATGCGTCAATCGTATGTGAATGCTGGTCGCTATACTGCTGTCGATTCGATACTAACGCCGCCATCCAGTGTCGAC  1050
```

Figure 2b: Map and sequence of selector module *kanMX4* (Wach *et al.*, 1994). DNA sequence obtained from PCR product of yeast cells transformed with *kanMX4* module. Homologous sequence is over-lined.